NEW STRATEGIES FOR TARGETED CHRONIC MYELOID LEUKEMIA THERAPY: DISRUPTING BCR-ABL1 DIMERIZATION AND SECONDARY LEUKEMIA-SPECIFIC PATHWAYS

by

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STATEMENT OF DISSERTATION APPROVAL

The following faculty members served as the supervisory committee chair and members for the dissertation of David W. Woessner .

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ABSTRACT

Chronic myeloid leukemia (CML) is identified by the unique reciprocal chromosomal translocation involving BCR and ABL1, the fusion of which generates a constitutively active tyrosine kinase. Of critical importance for kinase function is oligomerization of multiple BCR-ABL1 proteins, facilitated by the N-terminal coiled-coil (CC) domain in BCR. While antineoplastic therapies have historically been dominated by small molecule drugs with a broad impact on cancer, recently there has been a shift toward small molecule targeted therapeutics, which was led by the development of imatinib. Imatinib, a tyrosine kinase inhibitor (TKI), was rationally developed for the treatment of CML. Although imatinib has been extremely successful in disease modification and increasing overall survival, it, like many of the subsequently developed TKIs, is subject to failure when mutations in the BCR-ABL1 kinase domain (the target of TKIs) occur, or the cell loses its dependence on the BCR-ABL1 protein. We have broken from the small molecule development track and instead focused on peptide-based inhibition of the upstream oligomerization event in CML pathogenesis.

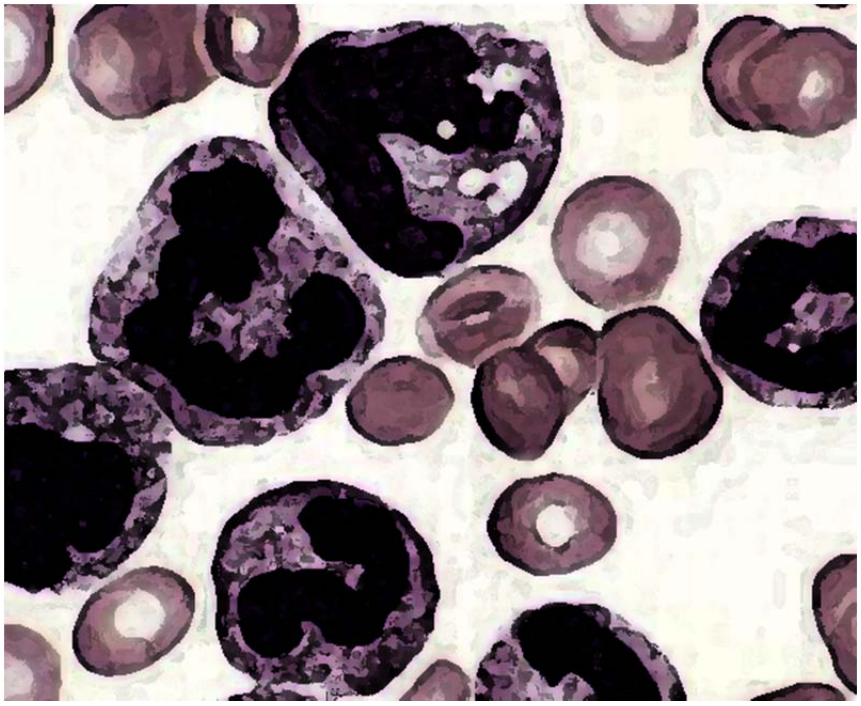
We previously described the anticancer activity of a dimerization inhibitor derived from the CC domain, called $CC^{mut2/3}$ (representing two different versions of coiled-coil inhibitors). Driven by the positive results in previous studies, we proposed the following overarching hypothesis: *Differential manipulation of domains within one BCR-ABL1 protein; or parallel manipulation of multiple pathways within one*

BCR-ABL1-containing cell will lead to a potent therapy which may overcome TKI-resistant disease. Here we examine this hypothesis to determine the efficacy of the $CC^{mut2/3}$ for broad-spectrum CML disease.

In one study we observed that use of the CC^{mut2} in concert with one of several selective leukemia-specific secondary pathway inhibitors enhances the apoptotic potential and limits the proliferative capacity of K562 BCR-ABL1-containing cells. Another study describes the broad anticancer inhibitory potential of CC^{mut3} in cells with varying mutational status in the BCR-ABL1 kinase domain. Finally we investigate the potential of CC^{mut3} in the context of human disease with a series of ex vivo inquires using patient samples. This dissertation focuses on demonstrating efficacy of $CC^{mut2/3}$ as a front-line CML therapy against several cell lines including those with wild-type and mutant BCR-ABL1.

To my loving wife Amy
and all those who encouraged me along the way.

Also,
for those waiting for a cure.



"Although progress is being made towards the development of a ‘global’ pan-BCR-ABL inhibitor that inhibits the full spectrum of identified imatinib-resistant BCR-ABL point mutants (including T315I), the potential for the evolution of new drug-resistant point mutations in BCR-ABL at crucial points that influence drug binding, protein expression or protein activity continues to exist. This prediction justifies the continued development of more potent BCR-ABL inhibitors with their own unique mutagenicity profiles, as well as the continued use of more than one BCR-ABL inhibitor in combination. It also warrants the use of specific signal transduction inhibitors in combination with BCR-ABL inhibitors to achieve highly efficacious therapy with a reduced potential for the development of drug resistance."

-Ellen Weisberg, Paul W. Manley, Sandra W. Cowan-Jacob,
Andreas Hochhaus & James D. Griffin.
-Nature Reveiws Cancer, 2007

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LIST OF ABBREVIATIONS

AA	Amino acid
ABD	Actin binding domain
ABL1	Abelson leukemia oncogene
ABR	Active BCR-related
AKT	Protein kinase B
ALL	Acute lymphoblastic leukemia
allo-SCT	Allogenic hematopoietic stem cell transplant
Alox5	Arachidonate 5-lipoxygenase
AP	Accelerated phase
Atg7	Autophagy-related protein 7
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3 related
BC	Blast crisis phase
BCR	Breakpoint cluster region
BOS	Bosutinib
c-Abl	Cellular ABL
CC	Coiled-coil
CC^{mut}	Mutated coiled-coil
CC^{mut2}	Coiled-coil mutant version 2
CC^{mut3}	Coiled-coil mutant version 3
CCyR	Complete cytogenetic response
CFC	Colony forming cell
CHR	Complete hematologic response
CML	Chronic Myeloid (myelogenous) Leukemia
CP	Chronic phase
CQ	Chloroquine
CXCR4	Chemokine (C-X-C motif) receptor 4
DAS	Dasatinib
DASISION	Dasatinib versus imatinib in ND CP-CML
DBD	DNA binding domain
DH	Dbl homology
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
EV	Empty vector
FDA	Food and Drug Administration
FISH	Fluorescence in situ hybridization

FOXO	Forkhead box O proteins
GAB2	GRB2-associated binding protein 2
GAP	GTP-ase-activating proteins
GEF	Rho-guanine nucleotide exchange factor
GFR	Growth factor receptor
GM	Granulocyte and macrophage
GRB2	Growth factor receptor 2
GTP	Guanosine triphosphate
GTPase	GTP-hydrolyzing enzyme
HDAC	Histone deacetylase
Hh	Hedgehog
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
IFN-α	Interferon-alpha
IM	Imatinib
IRIS	International Randomized Study of Interferon and STI571
JAK	Janus kinase
LSC	Leukemia stem cell
LS-CPP	Leukemia-specific cell penetrating peptide
MCyR	Major cytogenetic response
MMR	Major molecular response
mTOR	Molecular target of rapamycin
MUC1	Mucin 1
ND	Newly diagnosed
NFAT	Nuclear factor of activated T-cells transcription factor
PACE	Ponatinib Ph+ ALL and CML Evaluation
PCR	polymerase chain reaction
PCyR	Partial cytogenetic response
PDGFR	Platelet-derived growth factor receptor
Ph	Philadelphia chromosome
PH	Pleckstrin homology
PI3K	Phosphatidylinositol-3 kinase
PML	Promyelocytic leukemia
PON	Ponatinib
Pon	Ponatinib
R	Resistant
RAC1	Ras-related GTP-binding protein
RNAi	RNA interference
SDF1	Stromal cell-derived factor 1
SH2/3	SRC homology 2/3
Smo	Smoothend
SOS	Son of sevenless
START	Src/ABL Tyrosine kinase inhibition Activity Research Trials of dasatinib
STAT	Signal transducers and activators of transcription
TCO	Technology commercialization office
TKI	Tyrosine Kinase Inhibitor

VEGFR	Vascular endothelial growth factor receptor
Yap	Yes associated protein 1
ZIL	Zileuton

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CHAPTER 1

BACKGROUND AND SIGNIFICANCE

Summary

Targeted small molecule therapeutics for treatment of chronic myeloid leukemia (CML) are now plentiful, yet still not curative, and are prone to resistance. The first orally available targeted tyrosine kinase inhibitor, imatinib (Gleevec®), for cancer therapy was developed for CML (1). Reaching that milestone represented a paradigm shift in treatment and outcome of this disease. Specifically, imatinib transformed a 37.7% 5-year survival rate (2) to 89% for patients who received this new drug as initial therapy following diagnosis (3). In the last decade since the initial adoption of imatinib (Gleevec®) as front-line therapy for CML, four other targeted tyrosine kinase inhibitors (TKIs) have been approved for CML treatment by the United States Food and Drug Administration (FDA): second (dasatinib (Sprycel®); nilotinib (Tasigna®); bosutinib (Bosulif®)) and third (ponatinib (Iclusig®)) generation drugs have all been developed to address resistance to previous-generation drugs (4-7). While each new small molecule drug is able to address more and different mutant forms of the oncogenic protein, resistance to single-agent small molecule therapy continues to be a problem (8, 9).

All current targeted therapies for CML address the aberrant and constitutive ABL tyrosine kinase activity of the oncogenic fusion protein BCR-ABL1, the causative

transforming event in CML (10, 11). Surprisingly, little attention has been given to the N-terminal fusion partner BCR which harbors the coiled-coil dimerization domain. Dimerization of BCR-ABL1 is an upstream and necessary event that leads to ABL1 tyrosine kinase phosphorylation through transactivation (12). In fact, wild-type BCR-ABL1 proteins lacking the N-terminal dimerization domain do not result in an oncogenic phenotype in vitro. We recently designed a mutated coiled-coil (CC) domain derived from the wild type dimerization domain of BCR-ABL1 (CC^{mut}) that competitively interacts with endogenous BCR-ABL1 to disrupt downstream oncogenic pathways (13, 14). The design and construction of these constructs for BCR-ABL1 inhibition will be discussed in some detail in this introductory chapter. Additionally, some initial testing in BCR-ABL1-containing cell lines will be presented.

The following chapter will discuss the clinical course of CML including historical, current standard-of-care, and new strategies in development for disease treatment. A common thread throughout this work will be the topic of disease resistance – this includes both BCR-ABL1-dependent resistance and BCR-ABL1-independent resistance. Briefly, BCR-ABL1-dependent resistance is the phenomenon that occurs when a change within the BCR-ABL1 molecule, typically a mutation within the tyrosine kinase domain, confers resistance to a therapeutic (15, 16). BCR-ABL1-independent resistance involves a change within the cell that harbors BCR-ABL1, where the cell is no longer addicted to/dependent on the oncogenic driver BCR-ABL1 to survive (17, 18). The latter poses a problem because these cells are difficult to irradicate with a single agent targeted therapy.

To address the early potential of coiled-coil combination therapy, selective

secondary agents were tested to determine their ability to enhance killing of BCR-ABL1 containing cells. Secondary agents were chosen on the ability to circumvent damage to normal hematopoietic cells, which becomes important in managing the health of an individual with CML (19-22). This work is discussed in detail in Chapter 3. Finally, the potential of the CC^{mut} to overcome BCR-ABL1-dependent resistance is also addressed. Because the main driver of TKI development has been to develop a drug that is more effective against multiple possible BCR-ABL1 mutants, the activity of the CC^{mut} against these mutants is examined in Chapter 4.

Background

The Breakpoint Cluster Region

Prior to its discovery as an associated gene partner of the BCR-ABL1 fusion gene, little was known about the breakpoint cluster region (BCR) gene or the biologic ramifications of its protein activity. BCR is classified by its ability to activate guanosine triphosphate (GTP) hydrolyzing enzymes (GTPases), also known as a GTP-ase-activating proteins (GAPs) (23). Specifically, BCR is a GAP for the Ras-related GTP-binding protein RAC1 (23). Transient RAC1 interaction is dependent on a GTP-bound state within a GAP domain (24). The RAC-GAP domain is found in the C-terminus of full length BCR (approximately 160 kDa) and is lost in most recombination events with ABL (partially retained in p230 BCR-ABL) (25). Moving backward from the C-terminus, BCR also contains a site for Rho-guanine nucleotide exchange factor (GEF) binding within the DH/PH domain, which in addition to promoting GEF function also facilitates protein-protein interactions (26-28). The remaining functional properties arise from the CC

domain, which will be discussed more extensively later in this chapter, and the serine/threonine (S/T) kinase domain. The S/T kinase domain is responsible for both auto- and transphosphorylation activity (29). Most importantly, phosphorylation of Y-177 enables the binding of growth factor receptor 2 (GRB2) and other scaffolding proteins which initiate several signaling cascades important in cell survival, proliferation and transcription (30). It is through autophosphorylation of this domain that the activity of BCR, in the context of a BCR-ABL1 fusion protein, is switched (31, 32).

While the interactions and function of BCR described above are known, the predominant function of BCR in the context of human health and disease (beyond CML) is not well understood. A myriad of reports suggest varying roles for BCR in gross cell events and specific physiological activities. Kinase activity of BCR is hypothesized to be responsible for trafficking of growth factor receptors (GFR) including epidermal GFR (EGFR), as well as mediating transcriptional activity by interactions with peroxisome-proliferator-activated receptor gamma (33, 34). Functions of BCR and a homologous protein, active BCR related (ABR), are now being described together in some studies (35). BCR and ABR appear to be important in inflammatory responses, memory- and learning-related synaptic plasticity, and are potentially important in bipolar disorder (36-38). While some of these functional properties described are mediated by known domains (e.g., synaptic plasticity regulation by the RAC-GAP domain), the larger picture of how each domain is important in the context of these biologic process still remains largely a mystery.

Abelson Tyrosine-Protein Kinase 1

The cellular Abelson Protein Tyrosine Kinase 1 (c-Abl) is encoded by the ABL1 gene and produces a 140 kDa protein classified as a nonreceptor tyrosine kinase (39). Perhaps most interesting is the reciprocal role of a tightly regulated proapoptotic protein tyrosine kinase 1 vs. the constitutively active antiapoptotic and oncogenic BCR-ABL1 within the cell (40). Of the two c-Abl isoforms (1a and 1b) both contain an N-terminal "cap" region that in the Abl 1b isoforms is myristoylated (41). Myristoylation along with autoinhibition, molecular interactions, a multitude of protein interactions, and specific subcellular localization contribute to the spatiotemporal regulation of proapoptotic function of c-Abl (39, 42-44). DNA damage is a key trigger responsible for activation of c-Abl. Upon DNA damage, the PI3K-related protein kinases ATM and ATR depend on c-Abl-mediated control of cell cycle progression, apoptosis, and DNA repair. Signaling through c-Abl feedback dictates activation of ATM and ATR substrates, Chk2 and Chk1, respectively (45). Additionally, c-Abl relays signaling from ATM and ATR to pro-apoptotic p73, p63, p53 and Yap (46, 47). In addition to the initiation and propagation of the DNA-damage response, c-Abl plays a direct role in regulating cleavage of caspases in both effector (3, 7) and initiator (8, 9) classes (48-51).

Chronic Myeloid Leukemia

BCR-ABL1

The acquired BCR-ABL1 rearrangement is formed through a reciprocal translocation between BCR (breakpoint cluster region) and ABL1 (c-abl oncogene 1) t(9;22) [q34.1;q11.21] in the hematopoietic stem cell (HSC) (10, 11, 52, 53). This

recombination results in a lengthened chromosome 9 (containing ABL1-BCR) and a shortened chromosome 22 (with the coding region for BCR-ABL1), also known as the Philadelphia (Ph) chromosome (Figure 1.1) (54, 55). This single event is responsible for transforming a normal HSC into a leukemia stem cell (LSC), and the expression of BCR-ABL1, but not ABL1-BCR, within the LSC is able to confer a proliferative advantage to the LSC and its daughter cells over normal hematopoietic cells (10, 52, 56).

While this work will focus on BCR-ABL1 primarily in the context of CML (the Ph chromosome is present in the vast majority of cases, but the BCR-ABL1 translocation is missing in patients with chronic myelomonocytic leukemia or Ph-negative CML), it is also important to note that the Ph chromosome also appears in approximately 25% of acute lymphoblastic leukemia (ALL) cases (57-59). The breakpoint between BCR and ABL1 primarily forms a 210 kDa protein in CML while a 190 kDa protein is seen in the majority of ALL cases (60). Although the mechanism behind the differential generation of BCR-ABL1 breakpoints is unknown, recent work does suggest there are different mechanisms which generate the p190 lymphoid (ALL) vs. p210 myeloid (CML) breakpoints (60). While further discussion of the breakpoint mechanism is beyond the scope of this work, it is important to note that both p190 and p210 BCR-ABL1 contain the BCR N-terminal dimerization domain and the ABL1 tyrosine kinase domain.

The BCR-ABL1 fusion oncogene is transcribed and translated into the constitutively active fusion protein tyrosine kinase BCR-ABL1 protein containing the following domains depicted in Figure 1.2. First, the main topic of the following chapters, the coiled-coil dimerization domain (CC), occupies the N-terminus of BCR. Next, a protein serine/threonine kinase domain (S/T kinase) is followed by the Dbl

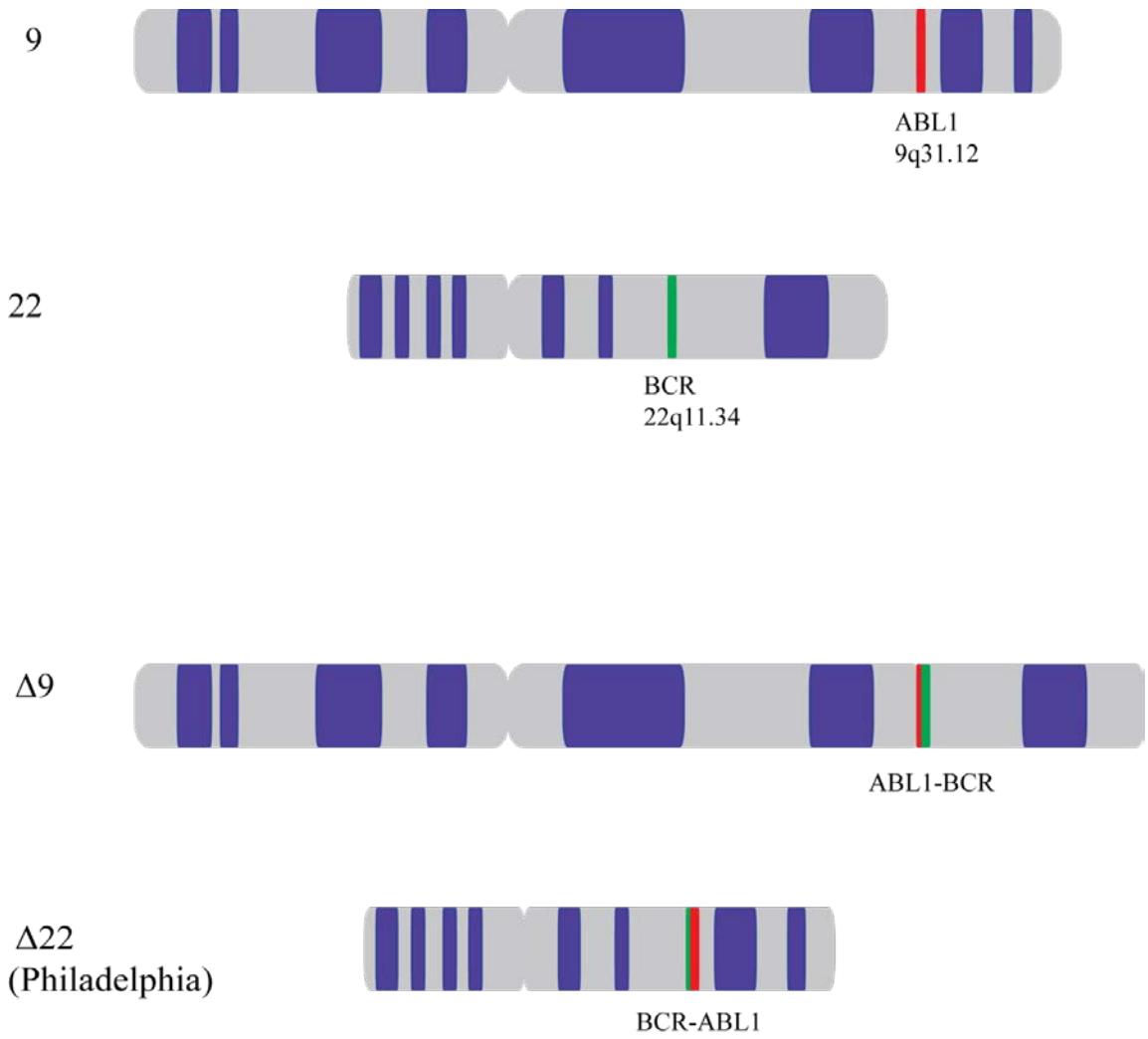

Figure 1.1 The chromosomal rearrangement of BCR-ABL1. ABL1 resides on the long arm of normal chromosome 9, while BCR is located on the long arm of chromosome 22. Following a reciprocal translocation, changed chromosome 9 (Δ9) is lengthened and now contains an ABL1-BCR fusion product. Changed chromosome 22 (Δ22) is shortened, called the Philadelphia chromosome, and harbors the oncogene BCR-ABL1.

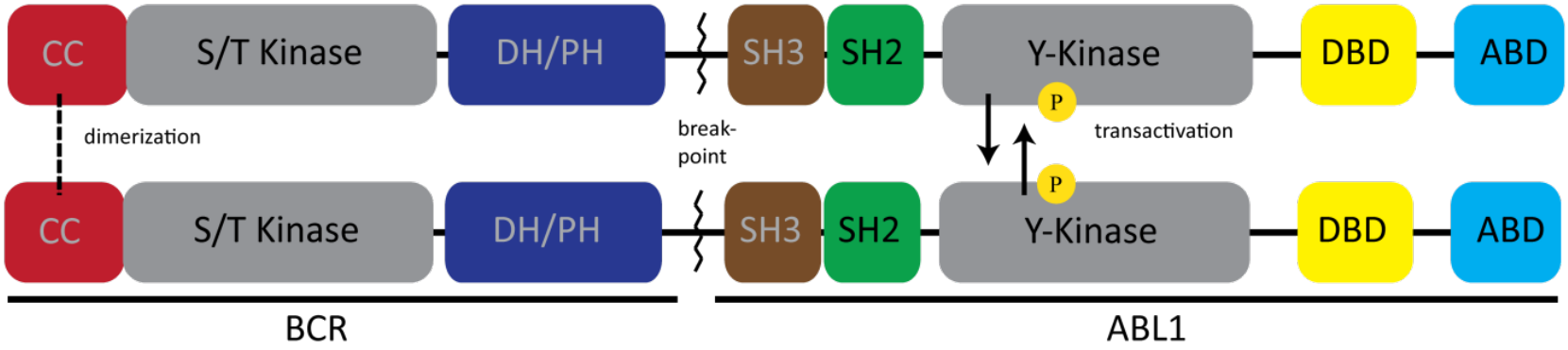

Figure 1.2 The domains and activation of BCR-ABL1. The fusion of BCR and ABL1 results in the C-terminal deletion of BCR and N-terminal truncation of ABL1. The breakpoint between BCR and ABL1 is shown for clarity. The resulting fusion protein contains a CC dimerization domain in BCR which is able to mediate the transactivation of the Y-kinase in ABL1.

Abbreviations: CC, coiled coil; S/T Kinase, serine/threonine kinase; DH/PH, Dbl/Pleckstrin-Homology; SH3/2, Src-homology 3/2; Y-kinase, tyrosine kinase; DBD, DNA binding; ABD, actin binding.

homology/Pleckstrin homology domain (DH/PH). Following the breakpoint in BCR, the domains in ABL1 include the Src-homology domains 3/2 (SH3/SH2) and current focus of drug therapy, the protein tyrosine kinase domain (Y-kinase). Finally the C-terminus of ABL1 includes both a DNA-binding and actin-binding domain (DBD/ABD) (61, 62).

While normal function of c-Abl (the protein expressed from a normal chromosome 9) is regulated by an N-terminal "cap" of approximately 80 residues (63-65), fusion to BCR results not only in the truncation of this regulatory region, but also enables oligomerization of BCR-ABL1 by the CC domain (66, 67). This antiparallel dimerization of BCR-ABL1 will then stack with a second dimer pair to form a tetramer (12). This arrangement is responsible for the transphosphorylation of tyrosine residues in ABL1 (68). Additionally, the cellular localization of c-Abl changes from a primarily nuclear to exclusively cytoplasmic spatial localization upon BCR-ABL1 fusion (69). The culmination of a change in subcellular location and constitutive phosphorylation results in a greatly increased diversity of interaction partners which can associate with the tyrosine kinase domain. (69, 70).

Downstream Targets of BCR-ABL1

The constitutively activated BCR-ABL1 tyrosine kinase initiates signaling cascades from both BCR and ABL1. Though most of the focus thus far has indicated c-Abl aberrant kinase activity is the key oncogenic driver, BCR is not simply a bystander in the genesis of the CML disease state. BCR has several key residues for phosphorylation in the S/T kinase domain, the best studied of which is Y-177. Phosphorylation of c-Abl on Y-245 and Y-412 are required for kinase activity (63). Phosphorylation of c-Abl Y-

245, located between the SH2 and kinase domain of c-Abl, is responsible for the autophosphorylation event to activate BCR-mediated signaling in BCR-ABL1 (71). Upon phosphorylation of BCR Y-177, a high-affinity site for GRB2 is generated. GRB2 initiates scaffolding for GRB2-associated binding protein 2 (GAB2), and son of sevenless (SOS) binding (43). The consequence of this initiation event is strong activation of pro-survival and proliferation pathways. These pathways are activated through nodal proteins with a documented history of aberrant activity in cancer. These include phosphatidylinositol-3 kinase (PI3K), the protein kinase B (AKT) pathway (72), RAS (73, 74), and signal transducers and activators of transcription (STAT) proteins (75, 76), specifically STAT3/5, and Janus kinases (JAK1/2) (77, 78). The BCR-ABL1 fusion oncoprotein is responsible for constitutive kinase activity able to induce malignant transformation by disrupting cell proliferation and differentiation (74, 79-81), cell survival (82-85) and cell adhesion and migration (Figure 1.3) (86-88).

Disease Statistics, Treatment and Resistance

A complete discussion of clinical disease presentation, progression, as well as historic and future therapeutic development avenues can be found in Chapter 2. However, a brief overview will be presented here for clarity of the following sections in this chapter.

CML is characterized by three disease phases: chronic, accelerated, and blast crisis (89). More than 90% of patients are now diagnosed in an indolent chronic phase and generally present with constitutional symptoms, enlarged spleen, and leukocytosis. This phase, if left untreated, will transition to an accelerated disease phase in 2 years and

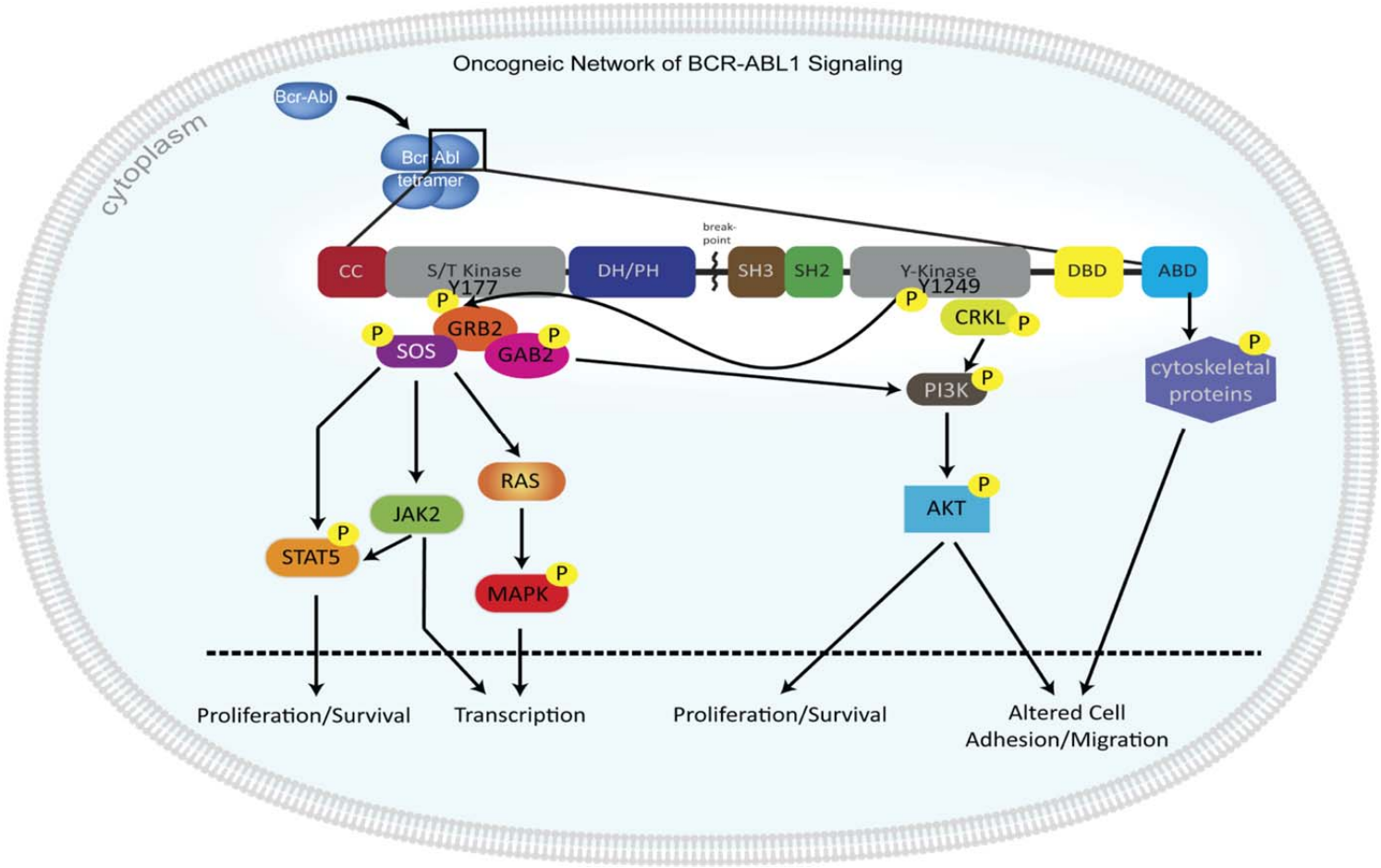

Figure 1.3. BCR-ABL1 transforms cells. The pleiotropic activity of BCR-ABL1 affects multiple proteins which are involved in transcription, survival and proliferation. Other pathways change cell adhesion and migration. Tetramerization of BCR-ABL1 leads to kinase activation in ABL1 (an ATP-dependent process). Kinase signaling from the Y-kinase domain ultimately results in the transformation of a tightly-regulated HSC signaling network to a dysregulated and always-on network in the LSC which provides a survival advantage.

then rapidly to an acute blast crisis phase, a hallmark of which is expansion of undifferentiated cells in the bone marrow and into the peripheral blood (89, 90). CML is fatal if not treated. While the exact molecular events leading to blast crisis phase disease are unknown, the proposed mechanism behind CML-progression is a process known as clonal evolution, represented by new chromosomal abnormalities, including gene amplification, mutations, and rearrangements (91, 92).

CML accounts for approximately 12% of all leukemias, and there are about 5,000 new cases of CML each year in the United States. The median age of diagnosis is 65 years old, while CML-related mortality also rises with age, peaking between 75 and 84 years of age (Figure 1.4A). Currently the only available curative therapy for CML is allogenic stem-cell transplantation. However, complications involving donor compatibility and treatment tolerance limit the feasibility of this therapy (93, 94). The alternative and first-line therapy for patients with CML is the drug class known as TKIs. Imatinib (IM) and other ABL1-targeted TKIs antagonize the causative oncogenic and constitutively active nonreceptor tyrosine kinase BCR-ABL1. Treatment with the decade-long TKI therapeutic veteran, imatinib, is initially effective in the majority of patients (48-51); however acquired resistance to this drug class may develop leading to disease progression (95). Second generation TKIs can effectively treat some IM-resistant strains of CML (4, 5, 96-99), but are ineffective against certain mutants (100). The most recent pan-BCR-ABL1 inhibitor, ponatinib, exhibits the most promising array of activity against all known BCR-ABL1 mutants. Ponatinib was developed specifically to address the elusive T315I, or “gatekeeper,” mutation in BCR-ABL (101). Although these single-agent small molecule inhibitors now have a strong foothold, experts theorize that

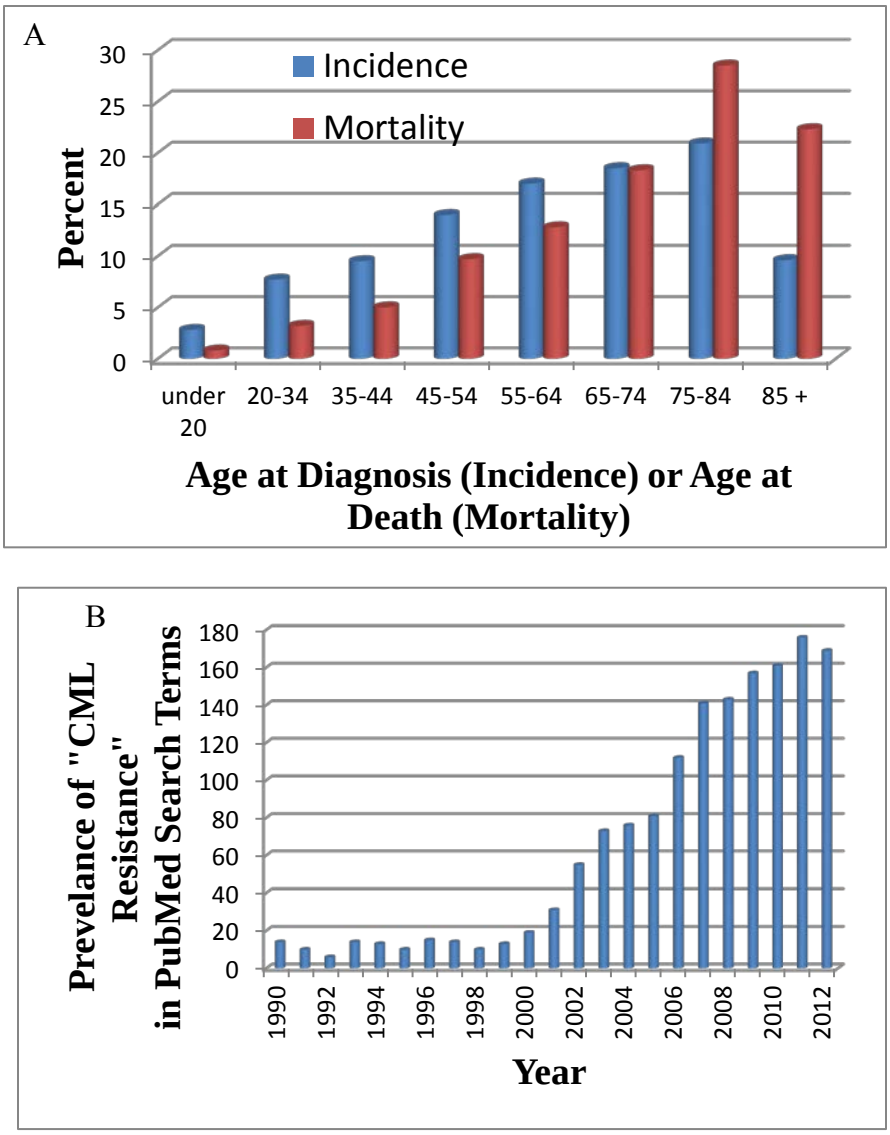

Figure 1.4 Incidence, mortality and resistance in CML. (A) Incidence and mortality of CML. CML incidence increases with age, with the median age at diagnosis of 65 years old. Disease-related mortality also increases with age with the highest percent occurring between 75-84 years. This data was compiled using the National Institutes of Health Surveillance Epidemiology and End Results (SEER) data from 1975-2009. (B**)** Increasing focus on CML resistance. Frequency of the keyword terms "CML" and "resistance" found in the U.S. National Library of Medicine, National Institutes of Health PubMed Database between 1990 and 2012.

resistance to this drug class is inevitable (9). However, work continues to explore the mechanism behind "mutational escape," and has led to a flurry of publications attempting to identify mechanisms of TKI resistance in CML (Figure 1.4B).

Disease persistence is a separate, but equally important subject pertaining to the population of Ph-positive LSCs, which, despite complete inhibition of BCR-ABL1, cells do not die. While TKIs can effectively kill peripheral Ph-positive cells, quiescent cells which reside in the bone marrow microenvironment cannot be completely eliminated (18, 70). This persistent population of cells is the probable origin of CML recurrence in the majority of patients who discontinue therapy (102, 103). It is important to note that TKIs are not curative, and ultimately their continued use increases CML prevalence.

To summarize, TKI-resistance and disease persistence can be broken down more simplistically into: 1) BCR-ABL1-dependent resistance; and 2) BCR-ABL1-independent resistance. Long-term single-agent disease management has been the preferred treatment strategy since the adoption of imatinib as the standard-of-care therapy. The second-generation TKIs dasatinib and nilotinib have first sought approval for imatinib-resistant indications before gaining accelerated approval for first-line indications. In all cases, drugs are administered over a consistent dose and constant schedule until there is indication of failure (104, 105). If a reason can be determined for the failure (e.g., detection of a kinase mutation in BCR-ABL1 by genotyping) the patient is transitioned to a more advanced TKI able to circumvent the problem or to a clinical trial where no therapies are available (106). Although theoretically BCR-ABL1-dependent resistance is solvable (107), the progression of disease will eventually outpace the lag between discovery and development, and there will be no advanced TKI to which to turn (108,

109).

In addition to BCR-ABL1, many key regulatory proteins have been implicated in CML persistence. Multiple-agent therapies are currently in clinical trials, beginning with TKI combinations, and are aimed at addressing the residual population of Ph-positive LSCs residing in the bone marrow (9). Some of these secondary agents broadly target all HSCs, similar to older broad-spectrum antineoplastic drugs. These key regulatory proteins, such as FOXO, PML, Hh/Smo (Hedgehog), and Wnt/β-catenin, may not be optimal targets since they regulate other critical HSC cellular functions in addition to promoting CML (110-113). Indeed, other combinations are "smarter" and attempt to knock out pathways specifically affected by the transition to BCR-ABL1 independence (114-118). Targeting key proteins that are either unique to or overexpressed in CML cells can be a better option, as a restricted expression profile provides an opportunity to explore the potential of RNAi therapies or specific small molecule inhibitors targeting these proteins or pathways. Several reports demonstrate enhanced potency using RNAi targeting drug efflux pumps, the autophagy pathway, or the Wnt/$Ca^{2+/}$NFAT signaling axis in combination with current TKIs (114, 119, 120). The synthetic lethality concept, as well as the multiple target/dual hit hypothesis are described in more detail in Chapter 2. Because the solution to eliminate all BCR-ABL-independent LSCs is still elusive, a viable secondary target must be identified and combined with a potent BCR-ABL1 inhibitor to develop the best possible therapeutic approach.

Manipulating the BCR-ABL1 Helix

The Coiled-Coil

The therapeutic potential of targeting the coiled-coil domain in BCR-ABL1 has yet to be fully explored in CML. Previous efforts to explore the importance of the CC domain in CML have centered on downstream events such as BCR or ABL1 phosphorylation (121-123). However, as previously discussed, the oligomerization of BCR-ABL1 is necessary for ABL1 transactivation, and absence of this domain in vitro causes loss of oncogenicity (12). Ruthardt et al. did explore the therapeutic potential of virally introducing a portion of the CC defined as the α2 helix (Figure 1.5) into a p185 BCR-ABL1 expressing cell line as a competitive inhibitor. They reported that this therapeutic: 1) interfered with the transformation potential of BCR-ABL1 and increased sensitivity to imatinib; and 2) potentially forced a BCR-ABL1 monomeric state and could effectively inhibit some imatinib-resistant BCR-ABL1 mutants with the exception of the T315I mutant (124, 125). Our laboratory has recently attempted to improve the therapeutic potential of the wild-type CC in hopes of producing a more potent and universal BCR-ABL1 inhibitor.

The CC consists of 72 amino acids and structurally folds into two distinct α-helixes joined by a short linker. The α1 helix is shorter and on the N-terminus is comprised of amino acids (AA) 5-15, while the α2 helix is longer (AA 28-67) and contains the antiparallel homodimerization interface (Figure 1.5). While the α2 helix is primarily responsible for dimerization through a hydrophobic core of interactions, the α1 helix can interact with its dimer partner to provide stabilization of the dimer pair through an aromatic core of interactions (126).

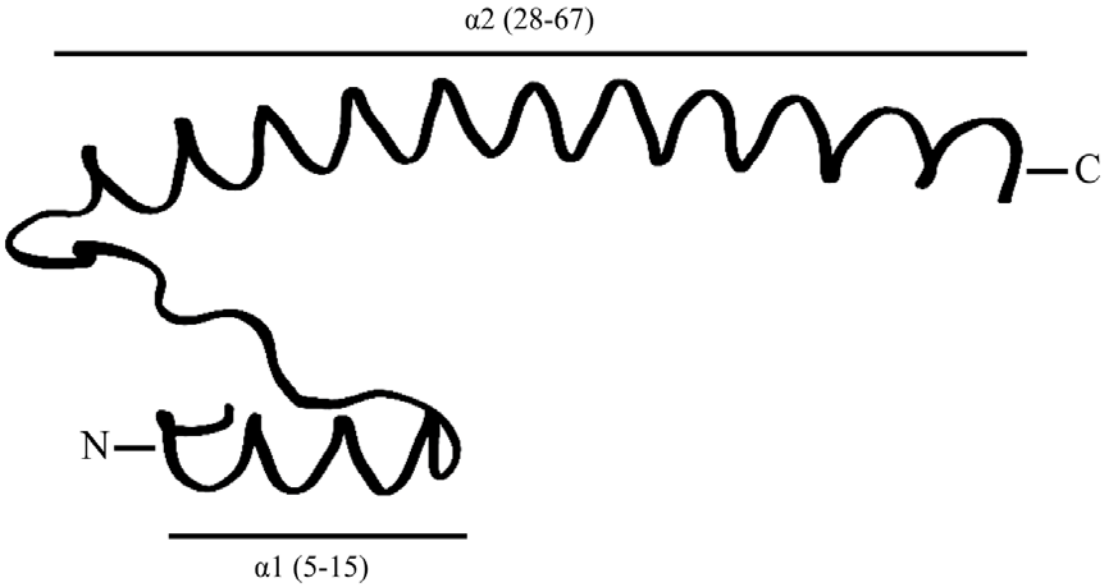

Figure 1.5 The coiled-coil domain. Comprised of 72 AA, the CC domain is structurally separated into two separate α-helixes. The α1 helix is close to the N-terminal portion while the α2 helix makes up the majority of the domain and is also primarily responsible for dimer interactions. This ribbon diagram of the CC was generated by James Robertson from modeling snapshots in AMBER11 using the 1K1F PDB structure of the BCR-ABL oligomerization domain.

The Mutant Coiled-Coil

Mut2

We recently designed a mutated coiled-coil (CC^{mut2}) derived from the wild-type dimerization domain of BCR-ABL1 which can bind endogenous BCR-ABL1 to disrupt oncogenic function (14). By introducing a competitive mutant CC to the endogenous full-length BCR-ABL1, we successfully limited the function of the BCR-ABL1 oncoprotein and transautophosphorylation of ABL1 tyrosine kinase. To improve oligomerization with BCR-ABL1 we mutated five residues to both increase the binding affinity for BCR-ABL1 (heterodimerization) and reduce binding to other CC^{mut2} molecules in the cell (homodimerization). The binding of the CC^{mut2} to wild-type Bcr-Abl itself is able to reduce proliferation, reduce phosphorylation of BCR-ABL1 and other downstream targets, and increase apoptosis.

The CC^{mut2} was first envisioned as a capture motif to bind BCR-ABL1 and, with the addition of some clever genetic engineering developed in our group called the protein switch (127), move the oncogenic protein from the cytoplasm to the nucleus (128). This was an important objective because artificial nuclear localization of BCR-ABL1 can be a potent driver of apoptosis (129, 130). However, we discovered that this technology could not overcome the tethering, primarily by the actin cytoskeleton, which held BCR-ABL1 in the cytoplasm (131).

Careful analysis of the helical wheel showing the α2 helix dimerization interface by Dixon (14) led to proposed mutations to strengthen this interaction. These were confirmed and amended following extensive computational modeling by Cheatham and Pendley (Figure 1.6) (14). The mutations which proved to be optimal based on modeling

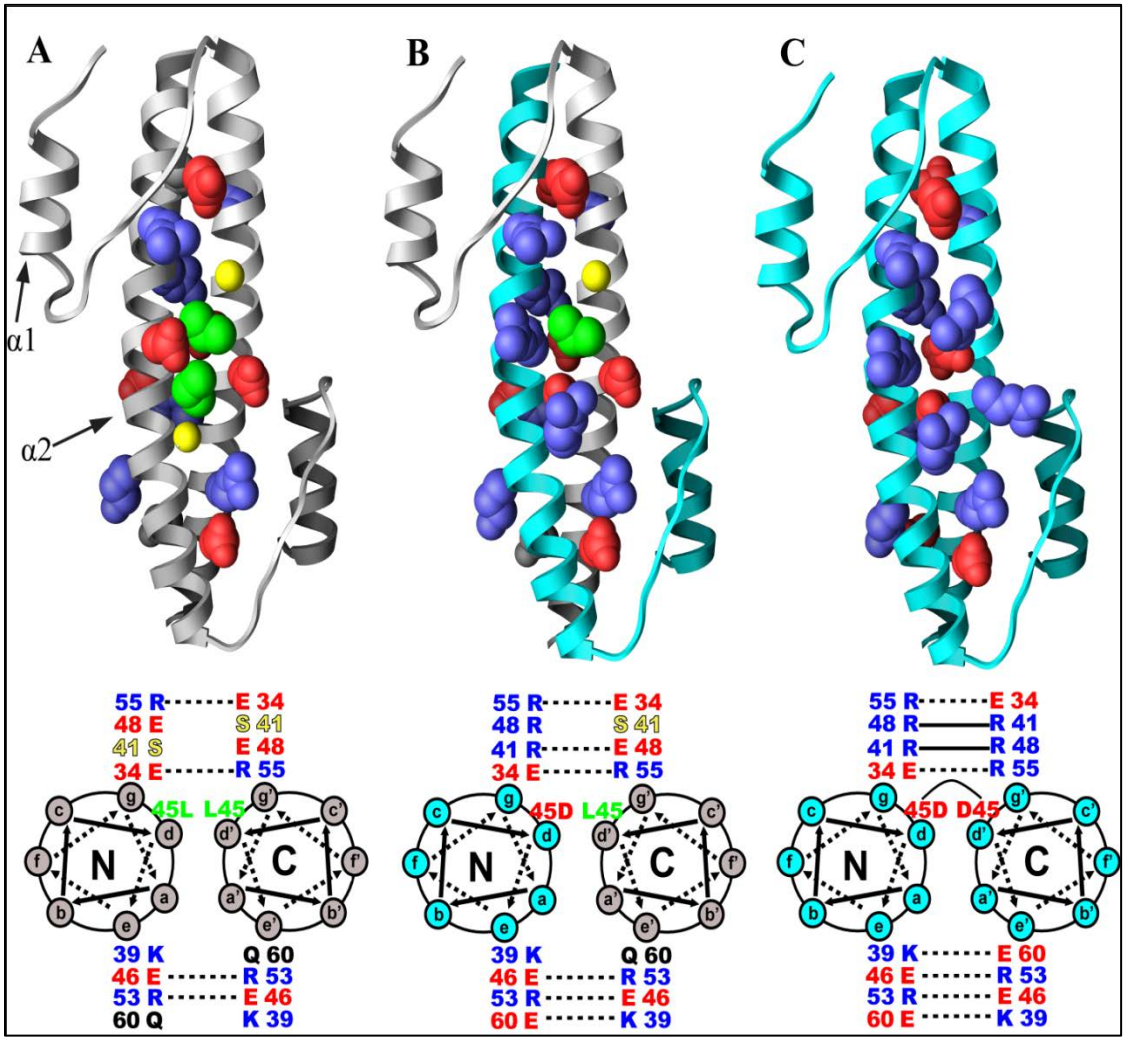

Figure 1.6 Mutant coiled-coil ribbon diagrams. Ribbon diagrams (with corresponding helical wheel diagram below) of wild-type homodimer (A), wild-type-CCmut2 heterodimer (B), andCCmut2-CCmut2 homodimer (C). Gray ribbons (ribbon diagrams) or dots (helical wheel diagrams) represent the wild-type coiled coil domain, and cyan represents CCmut2. The side chains of key residues (Glu-34, Lys-39, Ser/Arg-41, Leu/Asp-45, Glu-46, Glu/Arg-48, Arg-53, Arg-55, and Gln/Glu-60) are shown as red (acidic), blue (basic), green (hydrophobic), yellow (serine), or black (glutamine) spheres (ribbon diagrams) or font (helical wheel diagrams). Dotted lines indicate possible ionic interactions, and solid lines indicate charge-charge repulsions. Ribbon diagrams were generated with UCSF Chimera starting with the Bcr coiled coil domain crystal structure (Protein Data Bank code 1K1F).

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were C38A, S41R, L45D, E48R, and Q60E. While S41R, E48R and Q60E all increased electrostatic interactions, L45D stabilized the heterodimer pair, and C38A, in combination with the previously listed mutations, stabilized the heterodimer interaction over either homodimer pair (i.e., CC:CC or CC^{mut2}:CC^{mut2}).

Importantly, the introduction of CC^{mut2} in K562 cells significantly reduced the phosphorylation of the primary oncoprotein BCR-ABL1. This is especially interesting, because Beissert et al. reported significant activity with the wild-type α2 helix-CC, where we see little change between the full length CC and a GFP-control (124). However, this discrepancy could be a result of differing delivery methods (transfection vs. viral delivery by Ruthardt). In addition to the change in p-BCR-ABL, two downstream partners, CrkL and STAT5, also show a reduction in phosphorylation (Figure 1.7). Additionally, reduced cell proliferation, transformative potential, and increased apoptosis (as measured by caspase 3/7) were observed following administration of CC^{mut2}-expressing plasmids to K562 cells when compared to controls (Figure 1.8). These hallmarks of apoptosis and reduced proliferation were encouraging. Moreover, the proposed disfavoring of homodimer and promoting heterodimer formation were strongly supported. For a complete review of CC^{mut2} development and testing, the reader is referred to the original manuscript (14).

Mut3

Although we were encouraged by the efficacy of CC^{mut2}, we hypothesized additional improvements to the modified CC could be made, and this work was described in *Molecular Pharmaceutics* (13). A CC^{mut3} was created which consisted of the same five

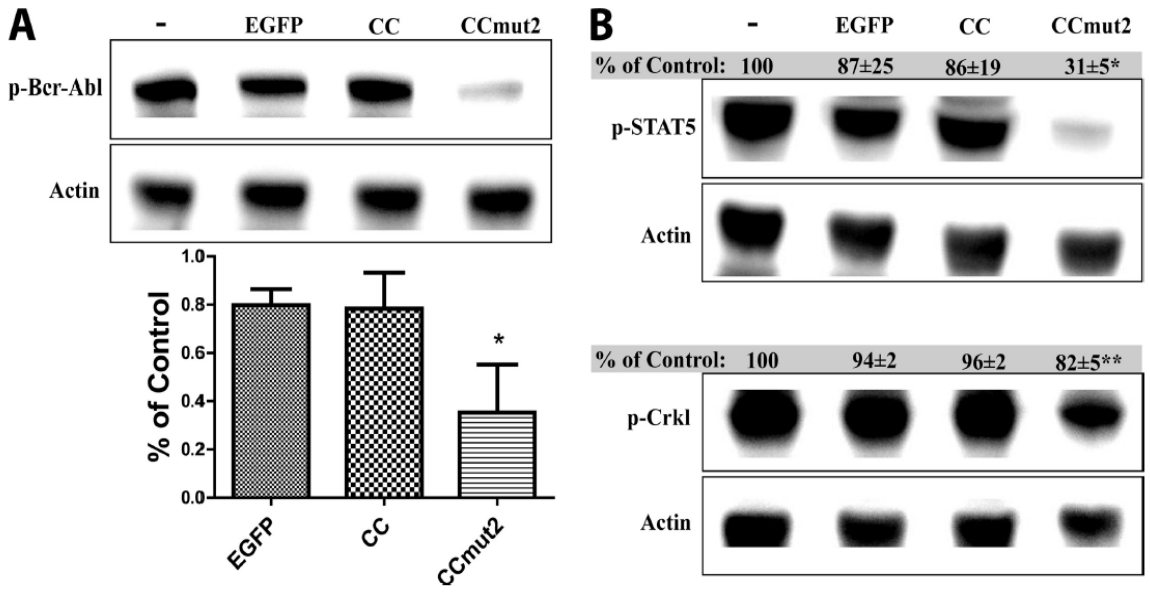

Figure 1.7 Phosphorylation mitigation by CCmut2. Representative images of Western blots to detect phosphorylated form of Bcr-Abl (A) and two substrates of Bcr-Abl, STAT5 and CrkL (B) are displayed here. The phosphorylation of Bcr-Abl is indicative of the tyrosine kinase activity and is shown to be decreased by the addition of CCmut2 (percentage of p-Bcr-Abl from untreated K562 cells is indicated graphically). The proteins STAT5 and CrkL are also phosphorylated when Bcr-Abl is active and are secondary indicators of the Bcr-Abl activity. Western blotting followed by densitometry was replicated three times on lysates from three separate transfections. The level of p-Bcr-Abl, as a percentage of the untreated cells, is shown graphically in A, and the level of p-STAT5 and p-CrkL (±S.D.) is indicated above the representative images. Statistical significance was determined using one-way ANOVA with Tukey's posttest (n=3). *,$p<0.05$; **,$p<0.01$ compared with cells transfected with EGFP. Error bars represent ±S.D.

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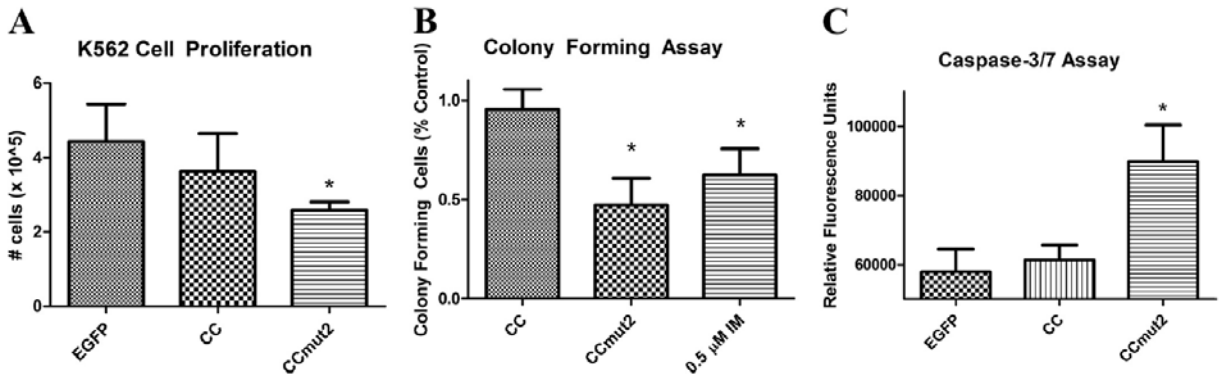

Figure 1.8 Decreased proliferation and apoptosis activation by CCmut2. Inhibition of Bcr-Abl through expression of CCmut2 results in decreased proliferation of K562 cells and activation of apoptosis. (A) Proliferation of K562 cells as determined by cell counts with trypan blue exclusion. (B) Proliferation of K562 cells as determined by colony forming assays. (C**)** Induction of apoptosis as measured through activation of caspase-3/7. For A–C, statistical significance was determined using one-way ANOVA withTukey's posttest. *, $p<0.01$; **, $p<0.001$ compared with control (cells transfected with pEGFP-C1). Error bars represent ± S.D. IM, imatinib mesylate.

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mutations described in the previous section (CC^{mut2}) with the addition of K39E in the CC. K39E is able to play two roles: first, K39 from the wild-type CC can form a salt bridge with E60 in CC^{mut3} promoting heteroligomerization; second, E39 in CC^{mut3} forms a charge-charge repulsion with E60 in CC^{mut3} further disfavoring homoligomerization (Figure 1.9). Although CC^{mut3} improved oligomerization properties in silico and in binding interaction assays, statistically significant biologic improvements in CML studies did not bear out these improvements. Nevertheless, we proceeded using CCmut3 in future biologic experiments as it demonstrates theoretical improvements that may be limited by assay sensitivity.

Statement of Objectives

The goal of the work discussed in the following studies is to advance feasibility studies for the CC^{mut} therapeutic. Although a timeline for delivery to patients is premature, preclinical efficacy is an essential hurdle to clear on the path to clinical development. This body of work was framed in the context of several hypotheses and corresponding aims, as follows:

1) **Hypothesis 1**: Inhibition of LSC-specific secondary pathways MUC1, Atg7, and Alox5 will induce apoptosis alone or in combination with the CC or CC^{mut}.
    a. *Aim 1*: Construction and validation of RNAi; combination treatment with RNAi.
        i. *Aim 1a*: Short hairpin RNAs (shRNAs) against MUC1, Atg7 and Alox5 transcripts will be constructed and validated using Western blotting.
        ii. *Aim 1b*: A synergistic or additive effect of RNAi with CC or CC^{mut} on apoptosis and proliferation will be assessed by transfecting both agents

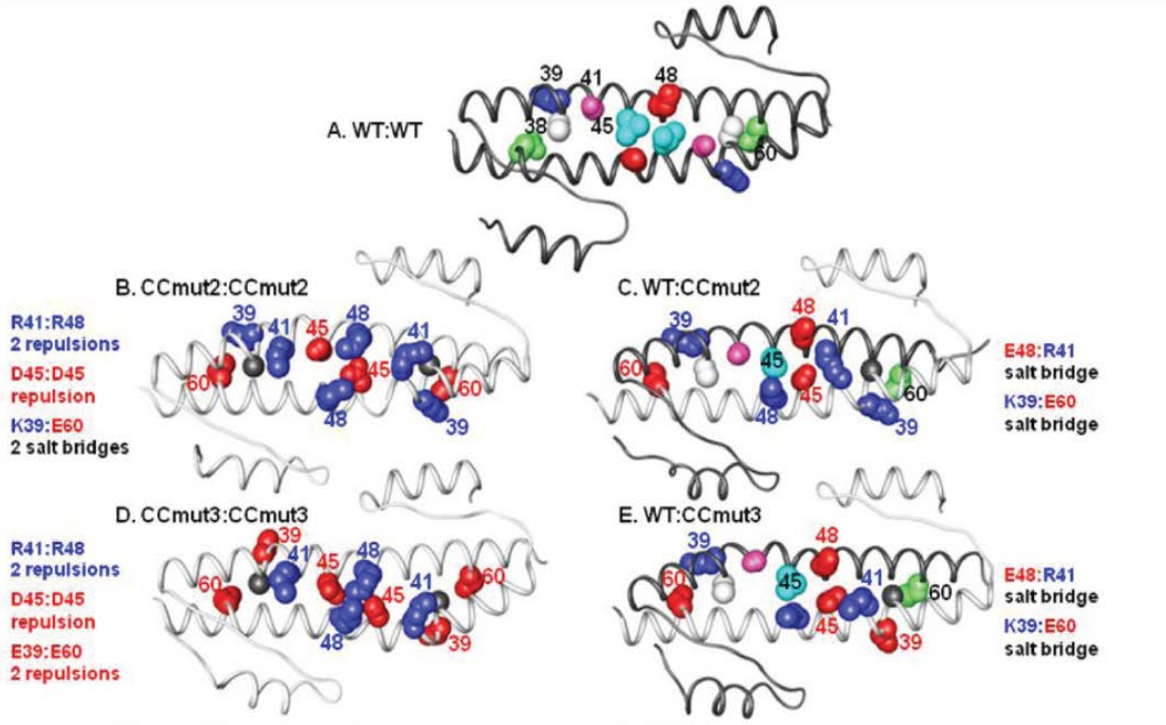

Figure 1.9 Ribbon diagrams of the coiled-coil domains. Gray ribbons indicate wild-type (WT) coiled-coil, white ribbons indicate mutant coiled-coils, and each homodimer (A, B, D, and F) or heterodimer (C, E, and G) is labeled above. Blue numbering/spheres indicates positively charged amino acid residue; red numbering/spheres indicates negatively charged amino acid residue. For the WT, white = C38, blue (+ chg) = K39, purple = S41, cyan (hϕ) = L45, red (− chg) = E48, green = Q60. For CC mutants, gray = C38A, red (− chg) = K39E, blue (+ chg) = S41R, red (− chg) = L45D, blue (+ chg) = E48R, red (− chg) = Q60E. (A) WT:WT homodimer. Only the top strand is numbered. (B) CCmut2:CCmut2 homodimer (CCmut2 contains C38A, S41R, L45D, E48R, Q60E mutations). The two R41:R48, and one D45:D45 charge-charge repulsion are shown, as well as the two sets of K39:E60 salt bridges. (C) WT:CCmut2 heterodimer. The E48:R41 and K39:E60 salt bridges are indicated. (D) CCmut3:CCmut3 homodimer (CCmut3 contains C38A, K39E, S41R, L45D, E48R, Q60E mutations). The two sets of K39:E60 salt bridges are now replaced with two sets of E39:E60 charge-charge repulsions. The two R41:R48 and one D45:D45 charge-charge repulsions are retained. (E) WT:CCmut3 heterodimers. CCmut3 may form E48:R41 and K39:E60 salt bridges with WT as illustrated.

into a BCR-ABL1-positive cell line (K562).

b. *Aim 2*: Drugs inhibiting the same target or pathway as RNAi will be combined with CC or CCmut to further investigate potential enhancements of apoptosis or proliferation.

2) **Hypothesis 2**: The efficacy of the CC^{mut} will not be affected by mutations in the tyrosine kinase domain that inactivate current TKIs.

a. *Aim 1*: A cell line derived from a mouse pro-B cell engineered to express p210-BCR-ABL1 (Ba/F3-p210) will be transfected with CC^{mut} to demonstrate efficacy equal to that of this construct in K562 cells. Parental Ba/F3 cells will be used to show lack of toxicity (as a control).

b. *Aim 2*: Kinase mutant BCR-ABL1 Ba/F3 cells will be tested to determine efficacy of the CC^{mut} by measuring proliferation, cell death, and transformation potential.

3) **Hypothesis 3**: The mutant coiled-coil therapeutic delivered by lentivirus as a gene-based therapy to primary CML patient samples in vitro will result in decreased proliferation and transformation potential.

a. *Aim 1*: Peripheral blood samples from newly diagnosed chronic phase CML will be enriched for $CD34^{+}$ cells and transduced with either CC^{mut} or a control empty vector.

b. *Aim 2*: Primary cells with or without BCR-ABL kinase mutations will be assessed for sensitivity to the CC^{mut} therapeutic.

These hypotheses and aims are discussed in the chapters that follow. Chapter 2 reviews the general disease course for CML as well as historical and current therapies.

Discussion of agents under development for CML and perspectives on curative strategies are highlighted as published in *The Cancer Journal*. Chapter 3 tackles Hypothesis 1 as published in *Molecular Pharmaceutics*. Chapter 4 addresses Hypotheses 2 and 3 and is in preparation for submission to *Leukemia*.

References

1. Cohen MH, Williams G, Johnson JR, Duan J, Gobburu J, Rahman A, Benson K, Leighton J, Kim SK, Wood R, et al. Approval summary for imatinib mesylate capsules in the treatment of chronic myelogenous leukemia. *Clin Cancer Res.* 2002;8(5):935-42.

2. National Cancer Institute NIoH. Surveillance epidemiology and end results web site. Http://seer.Cancer.Gov/statfacts/html/cmyl.Html.

3. Druker BJ, Guilhot F, O'Brien SG, Gathmann I, Kantarjian H, Gattermann N, Deininger MW, Silver RT, Goldman JM, Stone RM, et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med.* 2006;355(23):2408-17.

4. Talpaz M, Shah NP, Kantarjian H, Donato N, Nicoll J, Paquette R, Cortes J, O'Brien S, Nicaise C, Bleickardt E, et al. Dasatinib in imatinib-resistant philadelphia chromosome-positive leukemias. *N Engl J Med.* 2006;354(24):2531-41.

5. Kantarjian H, Giles F, Wunderle L, Bhalla K, O'Brien S, Wassmann B, Tanaka C, Manley P, Rae P, Mietlowski W, et al. Nilotinib in imatinib-resistant cml and philadelphia chromosome-positive all. *N Engl J Med.* 2006;354(24):2542-51.

6. Remsing Rix LL, Rix U, Colinge J, Hantschel O, Bennett KL, Stranzl T, Muller A, Baumgartner C, Valent P, Augustin M, et al. Global target profile of the kinase inhibitor bosutinib in primary chronic myeloid leukemia cells. *Leukemia.* 2009;23(3):477-85.

7. Cortes J, Talpaz M, Bixby D, Deininger M, Shah N, Flinn IW, Mauro M, O'Hare T, Hu S, Kan R, et al. A phase 1 trial of oral ponatinib (ap24534) in patients with refractory chronic myelogenous leukemia (cml) and other hematologic malignancies: Emerging safety and clinical response findings. *ASH Annual Meeting Abstracts.* 2010;116(21):210.

8. Schenone S, Bruno O, Radi M, and Botta M. New insights into small-molecule inhibitors of bcr-abl. *Med Res Rev.* 2011;31(1):1-41.

9. Woessner DW, Lim CS, and Deininger MW. Development of an effective therapy for chronic myelogenous leukemia. *Cancer J.* 2011;17(6):477-86.

10. Rowley JD. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and giemsa staining. *Nature.* 1973;243(5405):290-3.

11. Nowell PC, Jensen J, and Gardner F. Two complex translocations in chronic granulocytic leukemia involving chromosomes 22, 9, and a third chromosome. *Humangenetik.* 1975;30(1):13-21.

12. McWhirter JR, Galasso DL, and Wang JY. A coiled-coil oligomerization domain of bcr is essential for the transforming function of bcr-abl oncoproteins. *Mol Cell Biol.* 1993;13(12):7587-95.

13. Dixon AS, Miller GD, Bruno BJ, Constance JE, Woessner DW, Fidler TP, Robertson JC, Cheatham TE, 3rd, and Lim CS. Improved coiled-coil design enhances interaction with bcr-abl and induces apoptosis. *Mol Pharm.* 2012;9(1):187-95.

14. Dixon AS, Pendley SS, Bruno BJ, Woessner DW, Shimpi AA, Cheatham TE, 3rd, and Lim CS. Disruption of bcr-abl coiled coil oligomerization by design. *J Biol Chem.* 2011;286(31):27751-60.

15. O'Hare T, Eide CA, and Deininger MW. Bcr-abl kinase domain mutations, drug resistance, and the road to a cure for chronic myeloid leukemia. *Blood.* 2007;110(7):2242-9.

16. von Bubnoff N, Manley PW, Mestan J, Sanger J, Peschel C, and Duyster J. Bcr-abl resistance screening predicts a limited spectrum of point mutations to be associated with clinical resistance to the abl kinase inhibitor nilotinib (amn107). *Blood.* 2006;108(4):1328-33.

17. Nicholson E, and Holyoake T. The chronic myeloid leukemia stem cell. *Clin Lymphoma Myeloma.* 2009;9(S4):376-81.

18. Corbin AS, Agarwal A, Loriaux M, Cortes J, Deininger MW, and Druker BJ. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of bcr-abl activity. *J Clin Invest.* 2011;121(1):396-409.

19. Bishop JB, and Wassom JS. Toxicological review of busulfan (myleran). *Mutat Res.* 1986;168(1):15-45.

20. Cortés F JE, and Deininger M. *Chronic myeloid leukemia.* New York: Informa Healthcare; 2007.

21. Zhao C, Blum J, Chen A, Kwon HY, Jung SH, Cook JM, Lagoo A, and Reya T. Loss of beta-catenin impairs the renewal of normal and cml stem cells in vivo. *Cancer Cell.* 2007;12(6):528-41.

22. Irvine DA, Zhang B, Allan EK, Holyoake TL, Dorsch M, Manley PW, Bhatia R, and Copland M. Combination of the hedgehog pathway inhibitor lde225 and nilotinib eliminates chronic myeloid leukemia stem and progenitor cells *American Society of Hematology Annual Meeting.* 2009:#1428.

23. Diekmann D, Brill S, Garrett MD, Totty N, Hsuan J, Monfries C, Hall C, Lim L, and Hall A. Bcr encodes a gtpase-activating protein for p21rac. *Nature.* 1991;351(6325):400-2.

24. Kweon SM, Cho YJ, Minoo P, Groffen J, and Heisterkamp N. Activity of the bcr gtpase-activating domain is regulated through direct protein/protein interaction with the rho guanine nucleotide dissociation inhibitor. *J Biol Chem.* 2008;283(6):3023-30.

25. Deininger MW, and Druker BJ. Specific targeted therapy of chronic myelogenous leukemia with imatinib. *Pharmacol Rev.* 2003;55(3):401-23.

26. Chissoe SL, Bodenteich A, Wang YF, Wang YP, Burian D, Clifton SW, Crabtree J, Freeman A, Iyer K, Jian L, et al. Sequence and analysis of the human abl gene, the bcr gene, and regions involved in the philadelphia chromosomal translocation. *Genomics.* 1995;27(1):67-82.

27. Signorovitch JE, Wu EQ, Betts KA, Parikh K, Kantor E, Guo A, Bollu VK, Williams D, Wei LJ, and DeAngelo DJ. Comparative efficacy of nilotinib and dasatinib in newly diagnosed chronic myeloid leukemia: A matching-adjusted indirect comparison of randomized trials. *Curr Med Res Opin.* 2011;27(6):1263-71.

28. Chhatriwala MK, Betts L, Worthylake DK, and Sondek J. The dh and ph domains of trio coordinately engage rho gtpases for their efficient activation. *J Mol Biol.* 2007;368(5):1307-20.

29. Maru Y, and Witte ON. The bcr gene encodes a novel serine/threonine kinase activity within a single exon. *Cell.* 1991;67(3):459-68.

30. Ma G, Lu D, Wu Y, Liu J, and Arlinghaus RB. Bcr phosphorylated on tyrosine 177 binds grb2. *Oncogene.* 1997;14(19):2367-72.

31. Chu S, McDonald T, and Bhatia R. Role of bcr-abl-y177-mediated p27kip1 phosphorylation and cytoplasmic localization in enhanced proliferation of chronic myeloid leukemia progenitors. *Leukemia.* 2010;24(4):779-87.

32. Gillis LC, Berry DM, Minden MD, McGlade CJ, and Barber DL. Gads (grb2-related adaptor downstream of shc) is required for bcr-abl-mediated lymphoid leukemia. *Leukemia.* 2013;27(8):1666-76.

33. Alexis JD, Wang N, Che W, Lerner-Marmarosh N, Sahni A, Korshunov VA, Zou Y, Ding B, Yan C, Berk BC, et al. Bcr kinase activation by angiotensin ii inhibits peroxisome-proliferator-activated receptor gamma transcriptional activity in vascular smooth muscle cells. *Circ Res.* 2009;104(1):69-78.

34. Olabisi OO, Mahon GM, Kostenko EV, Liu Z, Ozer HL, and Whitehead IP. Bcr interacts with components of the endosomal sorting complex required for transport-i and is required for epidermal growth factor receptor turnover. *Cancer Res.* 2006;66(12):6250-7.

35. Heisterkamp N, Kaartinen V, van Soest S, Bokoch GM, and Groffen J. Human abr encodes a protein with gaprac activity and homology to the dbl nucleotide exchange factor domain. *J Biol Chem.* 1993;268(23):16903-6.

36. Oh D, Han S, Seo J, Lee JR, Choi J, Groffen J, Kim K, Cho YS, Choi HS, Shin H, et al. Regulation of synaptic RAC1 activity, long-term potentiation maintenance, and learning and memory by BCR and ABR RAC GTPase-activating proteins. *J Neurosci.* 2010;30(42):14134-44.

37. Cunnick JM, Schmidhuber S, Chen G, Yu M, Yi SJ, Cho YJ, Kaartinen V, Minoo P, Warburton D, Groffen J, et al. Bcr and abr cooperate in negatively regulating acute inflammatory responses. *Mol Cell Biol.* 2009;29(21):5742-50.

38. Masui T, Hashimoto R, Kusumi I, Suzuki K, Tanaka T, Nakagawa S, Suzuki T, Iwata N, Ozaki N, Kato T, et al. A possible association between missense polymorphism of the breakpoint cluster region gene and lithium prophylaxis in bipolar disorder. *Prog Neuropsychopharmacol Biol Psychiatry.* 2008;32(1):204-8.

39. Sirvent A, Benistant C, and Roche S. Cytoplasmic signalling by the c-abl tyrosine kinase in normal and cancer cells. *Biol Cell.* 2008;100(11):617-31.

40. Wang JY. Regulation of cell death by the abl tyrosine kinase. *Oncogene.* 2000;19(49):5643-50.

41. Zhou HX. How often does the myristoylated n-terminal latch of c-abl come off? *FEBS Lett.* 2003;552(2-3):160-2.

42. Hantschel O, and Superti-Furga G. Regulation of the c-abl and bcr-abl tyrosine kinases. *Nat Rev Mol Cell Biol.* 2004;5(1):33-44.

43. Pendergast AM, Quilliam LA, Cripe LD, Bassing CH, Dai Z, Li N, Batzer A, Rabun KM, Der CJ, Schlessinger J, et al. Bcr-abl-induced oncogenesis is mediated by direct interaction with the sh2 domain of the grb-2 adaptor protein. *Cell.* 1993;75(1):175-85.

44. Welch PJ, and Wang JY. Abrogation of retinoblastoma protein function by c-abl through tyrosine kinase-dependent and -independent mechanisms. *Mol Cell Biol.* 1995;15(10):5542-51.

45. Wang X, Zeng L, Wang J, Chau JF, Lai KP, Jia D, Poonepalli A, Hande MP, Liu H, He G, et al. A positive role for c-abl in atm and atr activation in DNA damage response. *Cell Death Differ.* 2011;18(1):5-15.

46. Meltser V, Ben-Yehoyada M, Reuven N, and Shaul Y. C-abl downregulates the slow phase of double-strand break repair. *Cell Death Dis.* 2010;1(e20.

47. Jin S, Kharbanda S, Mayer B, Kufe D, and Weaver DT. Binding of ku and c-abl at the kinase homology region of DNA-dependent protein kinase catalytic subunit. *J Biol Chem.* 1997;272(40):24763-6.

48. Nakamaki T, Okabe-Kado J, Yamamoto-Yamaguchi Y, Hino K, Tomoyasu S, Honma Y, and Kasukabe T. Role of mmtra1b/phospholipid scramblase1 gene expression in the induction of differentiation of human myeloid leukemia cells into granulocytes. *Exp Hematol.* 2002;30(5):421-9.

49. Gonfloni S, Di Tella L, Caldarola S, Cannata SM, Klinger FG, Di Bartolomeo C, Mattei M, Candi E, De Felici M, Melino G, et al. Inhibition of the c-abl-tap63 pathway protects mouse oocytes from chemotherapy-induced death. *Nat Med.* 2009;15(10):1179-85.

50. Kurokawa M, and Kornbluth S. Caspases and kinases in a death grip. *Cell.* 2009;138(5):838-54.

51. Bailey K, Cook HW, and McMaster CR. The phospholipid scramblase plscr1 increases uv induced apoptosis primarily through the augmentation of the intrinsic apoptotic pathway and independent of direct phosphorylation by protein kinase c delta. *Biochim Biophys Acta.* 2005;1733(2-3):199-209.

52. Bartram CR, de Klein A, Hagemeijer A, van Agthoven T, Geurts van Kessel A, Bootsma D, Grosveld G, Ferguson-Smith MA, Davies T, Stone M, et al. Translocation of c-ab1 oncogene correlates with the presence of a philadelphia chromosome in chronic myelocytic leukaemia. *Nature.* 1983;306(5940):277-80.

53. Nowell PC. The minute chromosome (phl) in chronic granulocytic leukemia. *Blut.* 1962;8:65-6.

54. The philadelphia chromosome and leukemia. *Can Med Assoc J.* 1961;84 (20): 1142.

55. Fitzgerald PH, Adams A, and Gunz FW. Chronic granulocytic leukemia and the philadelphia chromosome. *Blood.* 1963;21(183-96.

56. Anafi M, Gazit A, Gilon C, Ben-Neriah Y, and Levitzki A. Selective interactions of transforming and normal abl proteins with atp, tyrosine-copolymer substrates, and tyrphostins. *J Biol Chem.* 1992;267(7):4518-23.

57. Kurzrock R, Shtalrid M, Romero P, Kloetzer WS, Talpas M, Trujillo JM, Blick M, Beran M, and Gutterman JU. A novel c-abl protein product in philadelphia-positive acute lymphoblastic leukaemia. *Nature.* 1987;325(6105):631-5.

58. Walker LC, Ganesan TS, Dhut S, Gibbons B, Lister TA, Rothbard J, and Young BD. Novel chimaeric protein expressed in philadelphia positive acute lymphoblastic leukaemia. *Nature.* 1987;329(6142):851-3.

59. Burmeister T, Schwartz S, Taubald A, Jost E, Lipp T, Schneller F, Diedrich H, Thomssen H, Mey UJ, Eucker J, et al. Atypical bcr-abl mrna transcripts in adult acute lymphoblastic leukemia. *Haematologica.* 2007;92(12):1699-702.

60. Score J, Calasanz MJ, Ottman O, Pane F, Yeh RF, Sobrinho-Simoes MA, Kreil S, Ward D, Hidalgo-Curtis C, Melo JV, et al. Analysis of genomic breakpoints in p190 and p210 bcr-abl indicate distinct mechanisms of formation. *Leukemia.* 2010;24(10):1742-50.

61. Heisterkamp N, Stam K, Groffen J, de Klein A, and Grosveld G. Structural organization of the bcr gene and its role in the ph' translocation. *Nature.* 1985;315(6022):758-61.

62. Kin Y, Li G, Shibuya M, and Maru Y. The dbl homology domain of bcr is not a simple spacer in p210bcr-abl of the philadelphia chromosome. *J Biol Chem.* 2001;276(42):39462-8.

63. Pluk H, Dorey K, and Superti-Furga G. Autoinhibition of c-abl. *Cell.* 2002;108(2):247-59.

64. Smith KM, Yacobi R, and Van Etten RA. Autoinhibition of bcr-abl through its sh3 domain. *Mol Cell.* 2003;12(1):27-37.

65. Nagar B, Hantschel O, Young MA, Scheffzek K, Veach D, Bornmann W, Clarkson B, Superti-Furga G, and Kuriyan J. Structural basis for the autoinhibition of c-abl tyrosine kinase. *Cell.* 2003;112(6):859-71.

66. Quintas-Cardama A, and Cortes J. Molecular biology of bcr-abl1-positive chronic myeloid leukemia. *Blood.* 2009;113(8):1619-30.

67. Deininger MW, Goldman JM, and Melo JV. The molecular biology of chronic myeloid leukemia. *Blood.* 2000;96(10):3343-56.

68. Liu J, Campbell M, Guo JQ, Lu D, Xian YM, Andersson BS, and Arlinghaus RB. Bcr-abl tyrosine kinase is autophosphorylated or transphosphorylates p160 bcr on tyrosine predominantly within the first bcr exon. *Oncogene.* 1993;8(1):101-9.

69. Hazlehurst LA, Bewry NN, Nair RR, and Pinilla-Ibarz J. Signaling networks associated with bcr-abl-dependent transformation. *Cancer Control.* 2009;16(2):100-7.

70. O'Hare T, Deininger MW, Eide CA, Clackson T, and Druker BJ. Targeting the bcr-abl signaling pathway in therapy-resistant philadelphia chromosome-positive leukemia. *Clin Cancer Res.* 2011;17(2):212-21.

71. Sattler M, Mohi MG, Pride YB, Quinnan LR, Malouf NA, Podar K, Gesbert F, Iwasaki H, Li S, Van Etten RA, et al. Critical role for gab2 in transformation by bcr/abl. *Cancer Cell.* 2002;1(5):479-92.

72. Fresno Vara JA, Casado E, de Castro J, Cejas P, Belda-Iniesta C, and Gonzalez-Baron M. Pi3k/akt signalling pathway and cancer. *Cancer Treat Rev.* 2004;30(2):193-204.

73. Fernandez-Medarde A, and Santos E. Ras in cancer and developmental diseases. *Genes Cancer.* 2011;2(3):344-58.

74. Puil L, Liu J, Gish G, Mbamalu G, Bowtell D, Pelicci PG, Arlinghaus R, and Pawson T. Bcr-abl oncoproteins bind directly to activators of the ras signalling pathway. *EMBO J.* 1994;13(4):764-73.

75. Yu H, Pardoll D, and Jove R. Stats in cancer inflammation and immunity: A leading role for stat3. *Nat Rev Cancer.* 2009;9(11):798-809.

76. Warsch W, Kollmann K, Eckelhart E, Fajmann S, Cerny-Reiterer S, Holbl A, Gleixner KV, Dworzak M, Mayerhofer M, Hoermann G, et al. High stat5 levels mediate imatinib resistance and indicate disease progression in chronic myeloid leukemia. *Blood.* 2011;117(12):3409-20.

77. Walz C, Crowley BJ, Hudon HE, Gramlich JL, Neuberg DS, Podar K, Griffin JD, and Sattler M. Activated jak2 with the v617f point mutation promotes g1/s phase transition. *J Biol Chem.* 2006;281(26):18177-83.

78. Verma A, Kambhampati S, Parmar S, and Platanias LC. Jak family of kinases in cancer. *Cancer Metastasis Rev.* 2003;22(4):423-34.

79. Afar DE, Goga A, McLaughlin J, Witte ON, and Sawyers CL. Differential complementation of bcr-abl point mutants with c-myc. *Science.* 1994;264(5157):424-6.

80. Jiang Y, Zhao RC, and Verfaillie CM. Abnormal integrin-mediated regulation of chronic myelogenous leukemia cd34+ cell proliferation: Bcr/abl up-regulates the cyclin-dependent kinase inhibitor, p27kip, which is relocated to the cell cytoplasm and incapable of regulating cdk2 activity. *Proc Natl Acad Sci USA.* 2000;97(19):10538-43.

81. Sawyers CL. Molecular consequences of the bcr-abl translocation in chronic myelogenous leukemia. *Leuk Lymphoma.* 1993;11(2S):101-3.

82. Bedi A, Zehnbauer BA, Barber JP, Sharkis SJ, and Jones RJ. Inhibition of apoptosis by bcr-abl in chronic myeloid leukemia. *Blood.* 1994;83(8):2038-44.

83. Cortez D, Kadlec L, and Pendergast AM. Structural and signaling requirements for bcr-abl-mediated transformation and inhibition of apoptosis. *Mol Cell Biol.* 1995;15(10):5531-41.

84. McGahon A, Bissonnette R, Schmitt M, Cotter KM, Green DR, and Cotter TG. Bcr-abl maintains resistance of chronic myelogenous leukemia cells to apoptotic cell death. *Blood.* 1994;83(5):1179-87.

85. Cotter TG. Bcr-abl: An anti-apoptosis gene in chronic myelogenous leukemia. *Leuk Lymphoma.* 1995;18(3-4):231-6.

86. Gordon MY, Dowding CR, Riley GP, Goldman JM, and Greaves MF. Altered adhesive interactions with marrow stroma of haematopoietic progenitor cells in chronic myeloid leukaemia. *Nature.* 1987;328(6128):342-4.

87. Tabe Y, Jin L, Iwabuchi K, Wang RY, Ichikawa N, Miida T, Cortes J, Andreeff M, and Konopleva M. Role of stromal microenvironment in nonpharmacological resistance of cml to imatinib through lyn/cxcr4 interactions in lipid rafts. *Leukemia.* 2012;26(5):883-92.

88. Durig J, Rosenthal C, Elmaagacli A, Heyworth C, Halfmeyer K, Kasper C, Novotny J, and Duhrsen U. Biological effects of stroma-derived factor-1 alpha on normal and cml cd34+ haemopoietic cells. *Leukemia.* 2000;14(9):1652-60.

89. Bacher U, Kern W, Schnittger S, Hiddemann W, Schoch C, and Haferlach T. Blast count and cytogenetics correlate and are useful parameters for the evaluation of different phases in chronic myeloid leukemia. *Leuk Lymphoma.* 2005;46(3):357-66.

90. Perrotti D, Jamieson C, Goldman J, and Skorski T. Chronic myeloid leukemia: Mechanisms of blastic transformation. *J Clin Invest.* 2010;120(7):2254-64.

91. Naka K, Hoshii T, Tadokoro Y, and Hirao A. Molecular pathology of tumor-initiating cells: Lessons from philadelphia chromosome-positive leukemia. *Pathol Int.* 2011;61(9):501-8.

92. Verma D, Kantarjian H, Shan J, O'Brien S, Estrov Z, Garcia-Manero G, Koller C, Borthakur G, and Cortes J. Survival outcomes for clonal evolution in chronic myeloid leukemia patients on second generation tyrosine kinase inhibitor therapy. *Cancer.* 2010;116(11):2673-81.

93. Oyekunle A, Klyuchnikov E, Ocheni S, Kroger N, Zander AR, Baccarani M, and Bacher U. Challenges for allogeneic hematopoietic stem cell transplantation in chronic myeloid leukemia in the era of tyrosine kinase inhibitors. *Acta Haematol.* 2011;126(1):30-9.

94. Baccarani M, Cortes J, Pane F, Niederwieser D, Saglio G, Apperley J, Cervantes F, Deininger M, Gratwohl A, Guilhot F, et al. Chronic myeloid leukemia: An update of concepts and management recommendations of european leukemianet. *J Clin Oncol.* 2009;27(35):6041-51.

95. Melo JV, and Chuah C. Resistance to imatinib mesylate in chronic myeloid leukaemia. *Cancer Lett.* 2007;249(2):121-32.

96. Hochhaus A, Kantarjian HM, Baccarani M, Lipton JH, Apperley JF, Druker BJ, Facon T, Goldberg SL, Cervantes F, Niederwieser D, et al. Dasatinib induces notable hematologic and cytogenetic responses in chronic-phase chronic myeloid leukemia after failure of imatinib therapy. *Blood.* 2007;109(6):2303-9.

97. Weisberg E, Manley P, Mestan J, Cowan-Jacob S, Ray A, and Griffin JD. Amn107 (nilotinib): A novel and selective inhibitor of bcr-abl. *Br J Cancer.* 2006;94(12):1765-9.

98. Kantarjian HM, Giles F, Gattermann N, Bhalla K, Alimena G, Palandri F, Ossenkoppele GJ, Nicolini FE, O'Brien SG, Litzow M, et al. Nilotinib (formerly amn107), a highly selective bcr-abl tyrosine kinase inhibitor, is effective in patients with philadelphia chromosome-positive chronic myelogenous leukemia in chronic phase following imatinib resistance and intolerance. *Blood.* 2007;110(10):3540-6.

99. Redaelli S, Piazza R, Rostagno R, Magistroni V, Perini P, Marega M, Gambacorti-Passerini C, and Boschelli F. Activity of bosutinib, dasatinib, and nilotinib against 18 imatinib-resistant bcr/abl mutants. *J Clin Oncol.* 2009;27(3):469-71.

100. Mian AA, Schull M, Zhao Z, Oancea C, Hundertmark A, Beissert T, Ottmann OG, and Ruthardt M. The gatekeeper mutation t315i confers resistance against small molecules by increasing or restoring the abl-kinase activity accompanied by aberrant transphosphorylation of endogenous bcr, even in loss-of-function mutants of bcr/abl. *Leukemia.* 2009;23(9):1614-21.

101. Thomas M, Huang WS, Wen D, Zhu X, Wang Y, Metcalf CA, Liu S, Chen I, Romero J, Zou D, et al. Discovery of 5-(arenethynyl) hetero-monocyclic derivatives as potent inhibitors of bcr-abl including the t315i gatekeeper mutant. *Bioorg Med Chem Lett.* 2011;21(12):3743-8.

102. Chomel JC, Bonnet ML, Sorel N, Bertrand A, Meunier MC, Fichelson S, Melkus M, Bennaceur-Griscelli A, Guilhot F, and Turhan AG. Leukemic stem cell persistence in chronic myeloid leukemia patients with sustained undetectable molecular residual disease. *Blood.* 2011;118(13):3657-60.

103. Chu S, McDonald T, Lin A, Chakraborty S, Huang Q, Snyder DS, and Bhatia R. Persistence of leukemia stem cells in chronic myelogenous leukemia patients in prolonged remission with imatinib treatment. *Blood.* 2011;118(20):5565-72.

104. Snead JL, O'Hare T, Eide CA, and Deininger MW. New strategies for the first-line treatment of chronic myeloid leukemia: Can resistance be avoided? *Clin Lymphoma Myeloma.* 2008;8 Suppl 3(S107-17.

105. Jabbour E, Hochhaus A, Cortes J, La Rosee P, and Kantarjian HM. Choosing the best treatment strategy for chronic myeloid leukemia patients resistant to imatinib: Weighing the efficacy and safety of individual drugs with bcr-abl mutations and patient history. *Leukemia.* 2010;24(1):6-12.

106. Jabbour E, and Kantarjian H. Chronic myeloid leukemia: 2012 update on diagnosis, monitoring, and management. *Am J Hematol.* 2012;87(11):1037-45.

107. Rumpold H, and Webersinke G. Molecular pathogenesis of philadelphia-positive chronic myeloid leukemia - is it all bcr-abl? *Curr Cancer Drug Targets.* 2011;11(1):3-19.

108. Nestal de Moraes G, Souza PS, Costas FC, Vasconcelos FC, Reis FR, and Maia RC. The interface between bcr-abl-dependent and -independent resistance signaling pathways in chronic myeloid leukemia. *Leuk Res Treatment.* 2012;2012(671702.

109. Joppi R, Bertele V, and Garattini S. Orphan drug development is progressing too slowly. *Br J Clin Pharmacol.* 2006;61(3):355-60.

110. Naka K, Hoshii T, and Hirao A. Novel therapeutic approach to eradicate tyrosine kinase inhibitor resistant chronic myeloid leukemia stem cells. *Cancer Sci.* 2010;101(7):1577-81.

111. Naka K, Hoshii T, Muraguchi T, Tadokoro Y, Ooshio T, Kondo Y, Nakao S, Motoyama N, and Hirao A. Tgf-beta-foxo signalling maintains leukaemia-initiating cells in chronic myeloid leukaemia. *Nature.* 2010;463(7281):676-80.

112. Al Baghdadi T, Abonour R, and Boswell HS. Novel combination treatments targeting chronic myeloid leukemia stem cells. *Clin Lymphoma Myeloma Leuk.* 2012;12(2):94-105.

113. Zhao C, Chen A, Jamieson CH, Fereshteh M, Abrahamsson A, Blum J, Kwon HY, Kim J, Chute JP, Rizzieri D, et al. Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. *Nature.* 2009;458(7239):776-9.

114. Bellodi C, Lidonnici MR, Hamilton A, Helgason GV, Soliera AR, Ronchetti M, Galavotti S, Young KW, Selmi T, Yacobi R, et al. Targeting autophagy potentiates tyrosine kinase inhibitor-induced cell death in philadelphia chromosome-positive cells, including primary cml stem cells. *J Clin Invest.* 2009;119(5):1109-23.

115. Han W, Pan H, Chen Y, Sun J, Wang Y, Li J, Ge W, Feng L, Lin X, Wang X, et al. Egfr tyrosine kinase inhibitors activate autophagy as a cytoprotective response in human lung cancer cells. *PLoS One.* 2011;6(6):e18691.

116. Yin L, Ahmad R, Kosugi M, Kawano T, Avigan D, Stone R, Kharbanda S, and Kufe D. Terminal differentiation of chronic myelogenous leukemia cells is induced by targeting of the muc1-c oncoprotein. *Cancer Biol Ther.* 2010;10(5):483-91.

117. Chen Y, Li D, and Li S. The alox5 gene is a novel therapeutic target in cancer stem cells of chronic myeloid leukemia. *Cell Cycle.* 2009;8(21):3488-92.

118. Chen Y, Peng C, Sullivan C, Li D, and Li S. Novel therapeutic agents against cancer stem cells of chronic myeloid leukemia. *Anticancer Agents Med Chem.* 2010;10(2):111-5.

119. Gregory MA, Phang TL, Neviani P, Alvarez-Calderon F, Eide CA, O'Hare T, Zaberezhnyy V, Williams RT, Druker BJ, Perrotti D, et al. Wnt/ca2+/nfat signaling maintains survival of ph+ leukemia cells upon inhibition of bcr-abl. *Cancer Cell.* 2010;18(1):74-87.

120. Rumpold H, Wolf AM, Gruenewald K, Gastl G, Gunsilius E, and Wolf D. Rnai-mediated knockdown of p-glycoprotein using a transposon-based vector system durably restores imatinib sensitivity in imatinib-resistant cml cell lines. *Exp Hematol.* 2005;33(7):767-75.

121. He Y, Wertheim JA, Xu L, Miller JP, Karnell FG, Choi JK, Ren R, and Pear WS. The coiled-coil domain and tyr177 of bcr are required to induce a murine chronic myelogenous leukemia-like disease by bcr/abl. *Blood.* 2002;99(8):2957-68.

122. Tauchi T, Miyazawa K, Feng GS, Broxmeyer HE, and Toyama K. A coiled-coil tetramerization domain of bcr-abl is essential for the interactions of sh2-containing signal transduction molecules. *J Biol Chem.* 1997;272(2):1389-94.

123. Zhang X, Subrahmanyam R, Wong R, Gross AW, and Ren R. The nh(2)-terminal coiled-coil domain and tyrosine 177 play important roles in induction of a myeloproliferative disease in mice by bcr-abl. *Mol Cell Biol.* 2001;21(3):840-53.

124. Beissert T, Hundertmark A, Kaburova V, Travaglini L, Mian AA, Nervi C, and Ruthardt M. Targeting of the n-terminal coiled coil oligomerization interface by a helix-2 peptide inhibits unmutated and imatinib-resistant bcr/abl. *Int J Cancer.* 2008;122(12):2744-52.

125. Beissert T, Puccetti E, Bianchini A, Guller S, Boehrer S, Hoelzer D, Ottmann OG, Nervi C, and Ruthardt M. Targeting of the n-terminal coiled coil oligomerization interface of bcr interferes with the transformation potential of bcr-abl and increases sensitivity to sti571. *Blood.* 2003;102(8):2985-93.

126. Zhao X, Ghaffari S, Lodish H, Malashkevich VN, and Kim PS. Structure of the bcr-abl oncoprotein oligomerization domain. *Nat Struct Biol.* 2002;9(2):117-20.

127. Kakar M, Davis JR, Kern SE, and Lim CS. Optimizing the protein switch: Altering nuclear import and export signals, and ligand binding domain. *J Control Release.* 2007;120(3):220-32.

128. Dixon AS, Kakar M, Schneider KM, Constance JE, Paullin BC, and Lim CS. Controlling subcellular localization to alter function: Sending oncogenic bcr-abl to the nucleus causes apoptosis. *J Control Release.* 2009;140(3):245-9.

129. Vigneri P, and Wang JY. Induction of apoptosis in chronic myelogenous leukemia cells through nuclear entrapment of bcr-abl tyrosine kinase. *Nat Med.* 2001;7(2):228-34.

130. Aloisi A, Di Gregorio S, Stagno F, Guglielmo P, Mannino F, Sormani MP, Bruzzi P, Gambacorti-Passerini C, Saglio G, Venuta S, et al. Bcr-abl nuclear entrapment kills human cml cells: Ex vivo study on 35 patients with the combination of imatinib mesylate and leptomycin b. *Blood.* 2006;107(4):1591-8.

131. Preyer M, Vigneri P, and Wang JY. Interplay between kinase domain autophosphorylation and f-actin binding domain in regulating imatinib sensitivity and nuclear import of bcr-abl. *PLoS One.* 2011;6(2):e17020.

CHAPTER 2

DEVELOPMENT OF AN EFFECTIVE THERAPY FOR CHRONIC MYELOGENOUS LEUKEMIA

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REVIEW ARTICLE

Development of an Effective Therapy for Chronic Myelogenous Leukemia

David W. Woessner, BA, Carol S. Lim, PhD,† and Michael W. Deininger, MD, PhD‡*

Abstract: Targeted small-molecule drugs have revolutionized treatment of chronic myeloid leukemia (CML) during the last decade. These agents interrupt a constitutively active BCR-ABL, the causative agent for CML, by interfering with adenosine 5′ triphosphate–dependent ABL tyrosine kinase. Although the efficacy of tyrosine kinase inhibitors (TKIs) has resulted in overall survival of greater than 90%, TKIs are not curative. Moreover, no currently approved TKIs are effective against the T315I BCR-ABL variant. However, a new generation of TKIs with activity against T315I is on the horizon. We will highlight the clinical utility of historical CML therapeutics, those used today (first- and second-generation TKIs), and discuss treatment modalities that are under development. Recent advances have illuminated the complexity of CML, especially within the marrow microenvironment. We contend that the key to curing CML will involve strategies beyond targeting BCR-ABL because primitive human CML stem cells are not dependent on BCR-ABL. Ultimately, drug combinations or exploiting synthetic lethality may transform responses into definitive cures for CML.

Key Words: Chronic myeloid leukemia, BCR-ABL, tyrosine kinase inhibitors, drug resistance, synthetic lethality

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Chronic myeloid leukemia (CML) is one of the most extensively studied cancers, and a highly treatable disease with overall survival greater than 90% using current therapies.[1–3] Chronic myelogenous leukemia results from a reciprocal transl ocation between chromosomes 9 and 22 [t(9;22)(q34;q11)], which is thought to occur in a hematopoietic stem cell. The derivative chromosome 22, originally believed to be a shortened 22, is commonly referred to as Philadelphia chromosome (Ph). As a result of the translocation, fusions are formed between the breakpoint cluster region (BCR) gene on chromosome 22 and the Abelson oncogene (ABL) on chromosome 9. BCR-ABL, which resides on Ph, is critical to disease pathogenesis, whereas its reciprocal ABL-BCR does not seem to play any major role.[4,5] The BCR-ABL protein, a constitutively active tyrosine kinase, drives survival and growth of CML cells.[6] This tyrosine kinase activity was subsequently exploited for targeted CML therapy with the development of the first successful tyrosine kinase inhibitor (TKI) imatinib.[7] Although CML accounts for only 20% of all adult and 2.6% of childhood leukemias in the United States,[8] it has become a paradigm of successful cancer therapy based on a rational treatment approach.

Patients are typically diagnosed in the chronic phase of CML (CP-CML) and usually present with constitutional symptoms, splenomegaly, and left-shifted neutrophilic leukocytosis. However, at least in developed countries, the disease is frequently discovered when an abnormal "routine" blood count leads to a diagnostic workup. The chronic phase is characterized by an expansion of the myeloid cell compartment, with preserved terminal differentiation. In the absence of efficient therapy, there is inexorable progression to accelerated phase (AP) and blastic phase/blast crisis (BP or BC), which are characterized by a gradual or sudden loss of differentiation capacity, poor response to treatment, and short survival.[9]

During the first half of the 20th century, treatment was largely limited to splenic irradiation, which offered pain control but no survival benefit. Effective drug therapy for CML began in 1953 with oral busulfan, an alkylating agent. Busulfan's use was limited by significant myelosuppression, marrow fibrosis, and prolonged aplasia but remained the preferred therapy for almost 20 years and is still in use as part of conditioning regimens in allogeneic stem cell transplantation.[10] Hydroxyurea, an inhibitor of ribonucleotide reductase, was introduced into CML therapy in 1972 and improved median survival rates over busulfan from 44 to 58 months; however, neither therapy prevented progression to BC-CML.[11–13] Allogeneic hematopoietic stem cell transplant (allo-SCT), pioneered by the Seattle group in the mid-1970s, was the first therapy known to induce a state of Ph-negativity and is still considered the only therapy with the potential of curing CML. Incremental improvements to transplant technology, such as better supportive care and high-resolution HLA typing, led to greatly improved outcomes.[14] Today, treatment algorithms reserve allografting for patients with progression to AP/BC.[15–17]

Interferon α (IFN-α) entered the therapeutic space in the mid-1980s and was the first drug that induced a cytogenetic response.[18] The exact mechanism for the antileukemic effect is not known but may involve enhanced immune surveillance, modulation of hematopoiesis, and/or interleukin signaling, resulting in selective toxicity to the leukemic clone.[19,20] In randomized controlled trials, the 6-year survival for patients on IFN therapy was 50%, much superior to chemotherapeutics (29% at 6 years with either busulfan or hydroxyurea).[21,22] Subsequent studies explored the combination of IFN with cytarabine, which had previously shown some activity as a single-agent for CML. On the basis of a randomized comparison, this combination advanced to standard-of-care drug therapy in the mid-1990s.[23] Still, only a minority of patients achieved durable responses, and most patients eventually progressed to BC. Therefore, the treatment algorithm was to offer an allogeneic stem cell transplant to all eligible patients, leaving the majority—those without a suitable donor or with prohibitive comorbidities—with IFN as their best option.[24,25]

With the advent of imatinib and the second-generation TKIs dasatinib and nilotinib, small-molecule drugs have become the mainstay for first-line CML management.[26–29] The success

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of TKI therapy has drastically improved patient survival, and projections indicate that CML prevalence will continue to increase as a result. In fact, it has been estimated that there may be up to 250,000 CML patients in the United States in 2040.[30]

Tyrosine kinase inhibitors are very effective inhibitors of BCR-ABL kinase activity; second-generation agents are more potent, and they have expanded inhibition against various BCR-ABL mutants resistant to the first-generation drug, imatinib.[31] As we mark a decade of imatinib use, we have developed an understanding of disease response to these targeted agents, although many questions still remain. Will long-term BCR-ABL inhibition by TKIs eradicate all disease-causing cells, at least in some patients? If not, how can this be accomplished? Will it be possible for one compound to completely inhibit all BCR-ABL variants, including the T315I gatekeeper mutant? This review will discuss currently approved standard-of-care drugs and highlight promising novel agents. In addition, we will cover therapeutic roadblocks, such as targeting the bone marrow microenvironment and BCR-ABL–independent survival of CML stem cells.

FOOD AND DRUG ADMINISTRATION–APPROVED FIRST-LINE TKIS

Measuring Response

Disease stage is monitored using peripheral blood and marrow differentials, marrow cytogenetics, BCR-ABL detection by fluorescence in situ hybridization (FISH), and BCR-ABL copy number surveillance by quantitative real-time polymerase chain reaction (PCR). Normalization of blood counts and spleen size is termed *complete hematologic remission* (CHR) and is the earliest measure of response. Cytogenetic response is measured as the percentage of Ph+ karyotypes in 20 bone marrow metaphases. Zero Ph metaphases constitutes a complete cytogenetic response (CCyR); 1% to 35%, a partial response (PCyR); 30% to 65%, a minor response; and 66% to 95%, a minimal response.[32] Major cytogenetic response (MCyR) includes both CCyR and PCyR. A major molecular response is defined as a 3-log reduction of BCR-ABL messenger RNA compared with a standardized baseline as measured by quantitative real-time PCR.[33] For an excellent perspective on response to TKI therapy, please see the recent review by Radich.[34]

Imatinib

Imatinib mesylate (STI571/Gleevec; Novartis) is a competitive inhibitor of the adenosine 5′ triphosphate (ATP)–binding site of the BCR-ABL tyrosine kinase. Its development is regarded as a prototype for structure-based design of specifically targeted inhibitors.[35] Preclinical efficacy was described first in patient-derived BCR-ABL–expressing cells and finally in a mouse model expressing BCR-ABL–positive cells.[36] A phase I trial included an initial cohort of 83 patients. Despite dose escalation up to 1000 mg daily, the maximum tolerated dose was not achieved, and 400 mg/d was selected as an effective dose.[7] Clinical efficacy (phase II) studies were conducted for each disease phase (CP, AP, and BC) enrolling more than 1000 patients. Impressively, these studies confirmed or surpassed the efficacy seen in phase I but also confirmed that responses in AP/BC are less frequent and less durable.[37–39] The phase III International Randomized Study of Interferon and STI571 (IRIS) study demonstrated clear superiority of imatinib over IFN plus low-dose cytarabine for CP-CML. Specifically, at 18 months, freedom from progression to AP/BC was 96.7% in the imatinib group and 91.5% in the IFN group ($P < 0.001$), with a CCyR of 76.2% compared with 14.5%.[40] On the basis of the efficacy seen in these studies, imatinib gained approval from the US Food and Drug Administration (FDA) for the treatment of patients who had failed IFN (2001) and for newly diagnosed patients in 2003. Subsequent updates of the IRIS study at 60 months confirmed these results. Overall survival in the patients treated with first-line imatinib was 89%, a revolutionary improvement over previous IFN-based regimens. No survival difference was demonstrated compared with the IFN/cytarabine arm because most IFN patients crossed over to imatinib for intolerance or lack of efficacy.[41]

Single-center studies had suggested that increasing imatinib from 400 to 800 mg/d could improve response rates. However, randomized comparisons failed to confirm these initial results.[42] More recently, the German CML IV study showed a significant difference in the rate of mismatch repair (MMR) in favor of higher doses of imatinib. It has been suggested that the more flexible dosing regimen in this study led to an overall higher dose intensity and a superior result.[43] At this point, the standard dose of imatinib for newly diagnosed patients remains 400 mg daily, and the drug remains a viable option for newly diagnosed patients in the chronic phase.[42] Imatinib, however, falls short of effectively treating most patients in AP/BC.

Dasatinib

Inhibitors targeting Src kinases were the goal of Lombardo et al[44] when they discovered a dual-Src/ABL kinase inhibitor initially referred to as BMS-354825 and now known as dasatinib (Sprycel; Bristol-Myers Squibb). Dasatinib binds with high affinity to both ABL and the SRC kinase in the ATP-binding site, translating to an ABL inhibition potency 300 times that of imatinib in biochemical and cell proliferation assays. In addition to SRC-family kinases, c-KIT, PDGFR-α/β, and the ephrin receptor kinases are also inhibited by dasatinib.[45] Uniquely, this TKI binds ABL in both the active and inactive states, leading to a more complete inhibition regardless of protein confirmation.[46]

Dasatinib dose escalation studies were conducted in a cohort of 84 patients across all CML disease phases including a minority with Ph+ acute lymphoblastic leukemia (ALL). A maximum tolerated dose for dasatinib was not determined, but importantly, patients who enrolled after previous imatinib intolerance showed no similar toxicities.[47] Efficacy of this phase I trial established 70 mg twice daily as the optimal dose for further studies. The phase II trials for Src/ABL Tyrosine kinase inhibition Activity Research Trials of dasatinib (START) were conducted separately for each disease phase. Dasatinib demonstrated a robust and durable response in CP (CHR, 87%; MyCR, 52%) and a progression-free survival of 92% at 8 months.[48] Impressive responses were seen in AP (MCyR, 33%) and BC (MCyR, 31% myeloid and 50% lymphoid); however, these responses were much less durable than those in CP.[49,50] In 2006, the FDA granted approval of dasatinib at 70 mg twice daily for refractory CML patients. Further dose optimization studies led recommendations of 100 mg once daily for CP-CML,[51,52] whereas 70 mg twice daily remained the dose for advanced CML.[53]

Nilotinib

To overcome imatinib resistance, nilotinib (AMN107/Tasigna; Novartis) was rationally designed based on a thorough analysis of the ABL-imatinib complex to increase binding affinity. Nilotinib is more selective than imatinib, favoring ABL inhibition over the 2 other target kinases KIT and PDGFR.[54] Nilotinib is 10 to 50 times more potent than imatinib and is an inhibitor of many BCR-ABL mutants that are resistant to imatinib.[54,55] Phase I studies for nilotinib in imatinib-resistant CML or Ph+ ALL patients revealed significant activity in the chronic

phase (CHR, 92%; CCyR, 35%) and acceptable responses in the accelerated phase, whereas results in the blastic phase were disappointing, recapitulating the imatinib experience.[56] An administration of 400 mg twice daily emerged as the phase II dose. Subsequent phase II studies in CP and AP reported MCyR of 48% and 29%, respectively.[57,58] Nilotinib was approved in 2007 for CP and AP-CML. Recent follow-up of these patients indicates that nilotinib provides a rapid and durable response in these disease phases, especially in patients with prior suboptimal response to imatinib.[27,59]

Resistance to Currently Approved TKIs

Despite the promise of TKIs in treating CML, drug resistance does occur. Resistance can be primary (failure of a newly diagnosed patient to achieve satisfactory response to drug) or secondary/acquired (failure of a patient on treatment who initially responded to maintain this response). Tyrosine kinase inhibitor failure has been linked to mutations in the ABL kinase domain that impair drug binding, increased BCR-ABL expression, and changes in drug efflux transporters that result in low intracellular drug concentrations, particularly with imatinib.[60,61] These changes can occur during progression to advanced disease phases, but they do not, in and of themselves, cause progression.[1] In vitro mutagenesis screens have been used to profile TKIs. These studies revealed the broadest activity for dasatinib, followed by nilotinib, whereas imatinib has extensive gaps in coverage, consistent with clinical data.[62,63] On the basis of in vitro profiles, we and others have developed heat maps of predicted in vivo activity.[64] However, it is important to note that the in vivo response is more complex, involving additional parameters such as plasma protein binding and plasma peak and trough drug concentrations.[65] As a result, the correlation between in vitro predictions and clinical responses is relatively weak,[66,67] with the notable exception of the T315I mutant, which is resistant to all currently approved TKIs. This poses a significant challenge to therapy because the T315I mutation is reported to represent 15% to 20% of all mutations.[68]

Tyrosine kinase inhibitors have transformed a previously fatal disease into a manageable chronic condition, but drug discontinuation usually results in disease recurrence, even in patients with profound responses such as MMR or "PCR-undetectable" CML, although rare exceptions may exist.[69,70] Thus, drug treatment must continue indefinitely, a significant drawback to current TKI therapy. Consistent with these clinical observations, there is evidence that all 3 agents fail to eliminate primitive CML cells and that the bone marrow environment is a potential safe haven for these cells.[71] Taken together, this suggests that minimal residual disease may be beyond the reach of our current TKI-based therapeutic arsenal. This is often referred to as disease persistence.

Second-Generation TKIs in First-Line Therapy

Treatment advantages of second-generation TKIs over imatinib were suggested during phase II studies; additional trials comparing these inhibitors were quickly planned and executed. The phase III trial, Evaluating Nilotinib Efficacy and Safety in Clinical Trials—Newly Diagnosed Patients, compared nilotinib 300 or 400 mg twice daily and imatinib (400 mg once daily). After 1 year, MMR for either nilotinib dose (43%–45%) was nearly double that of imatinib, and CCyR was significantly higher in the nilotinib cohorts (78%–80% vs 65%).[28] In addition, nilotinib was superior in progression-free survival. As a result, the FDA granted accelerated approval of nilotinib in June 2010 for newly diagnosed CML patients.[72]

The Dasatinib versus Imatinib Study in Treatment-Naive CP-CML Patients (DASISION) trial tested dasatinib at 100 mg daily versus imatinib 400 mg daily in newly diagnosed chronic phase patients. This report indicated a comparable advantage as seen in the Evaluating Nilotinib Efficacy and Safety in Clinical Trials—Newly Diagnosed Patients trial regarding MMR for dasatinib over imatinib (46% vs 28%) and CCyR (77% vs 66%).[26] Progression-free survival was also improved, although the difference failed to reach statistical significance. Regulatory approval of dasatinib for newly diagnosed CP-CML patients was granted in October 2010.

Adverse Effects of Currently Approved TKIs

A comprehensive appreciation of TKI-related toxicities is beyond the scope of this review. Hematologic toxicity is common and correlates with disease state, being more frequent in patients with advanced disease compared with newly diagnosed patients. It is generally believed that this reflects the more limited reserve of normal hematopoiesis in patients with long-standing or more aggressive CML. Nonhematologic toxicity is diverse and dependent on the specific TKI. The good news is that these toxicities are largely nonoverlapping, which implies that cross-intolerance to all 3 approved TKIs is rare. For a comprehensive and detailed review of toxicity, the reader is referred to a recent review.[73] Importantly, annual updates of the IRIS study, as well as independent studies, confirmed the safety of long-term imatinib therapy in the sense that grades 3 to 4 toxicities are rare, and no new and unexpected adverse effects became apparent with longer follow-up.[41,74] The body of data available for dasatinib and nilotinib is more limited, and it will be important to remain vigilant as therapeutic time increases for these drugs.

NOVEL AGENTS

ATP-Competitive ABL Inhibitors Without Activity Against T315I

Several TKIs have been developed that exhibit a target spectrum similar to the approved drugs, although they are distinct in off-target effects. The most advanced of these drugs is bosutinib (SKI-606; Wyeth), originally developed as a Src kinase inhibitor.[75] Bosutinib has shown inhibitory activity in CML cell lines and primary cells and has demonstrated tumor regression in CML xenograft models. Unlike approved TKIs, bosutinib does not inhibit c-Kit or PDGFR.[76] Phase I and II studies revealed drug activity in patients who failed imatinib. However, as expected, efficacy in patients who failed a second-generation TKI was lacking. A phase III study did not meet the primary end point (ie, superior rates of CCyR at 12 months in comparison with imatinib 400 mg daily). Current speculation attributes lack of efficacy to insufficient dose intensity triggered by dose interruptions due to diarrhea, a common, but transient adverse effect that should have been managed with supportive care. Bosutinib could possibly add to the therapeutic armamentarium as another drug with a unique adverse effect profile. However, it does not address the problems of the T315I mutant and BCR-ABL–independent resistance. Overall, the future of bosutinib is unclear.[77]

T315I Active Inhibitors

The most advanced third-generation inhibitor of BCR-ABL is ponatinib (AP24534; Ariad).[78] Unlike all approved TKIs, ponatinib is effective against the T315I mutant as well as a large sample of other mutants previously detected in patients with clinical TKI resistance.[68] In vitro screens revealed no mutational vulnerabilities in BCR-ABL, suggesting that ponatinib may be

the first true "pan-BCR-ABL" TKI. This drug also inhibits other kinases including FLT3, FGFR, vascular endothelial growth factor receptor (VEGFR), c-Kit, and PDGFR.[79,80] Ponatinib showed significant activity in a phase I study of patients with Ph+ leukemia, mostly CML, who had failed other TKIs. Interestingly, responses were most impressive in patients with the T315I mutation, turning a poor prognostic factor into a favorable one.[81] Ponatinib is currently in phase II clinical trials (PACE trial, Ponatinib Ph+ ALL and CML Evaluation). PACE is a global, single-arm clinical study including patients in all disease phases of CML and Ph+ ALL. Given its activity against the T315I mutant, ponatinib may well replace nilotinib and dasatinib in salvage therapy. A phase III study for ponatinib in first-line therapy is in the planning stage.

Aurora kinases are serine/threonine kinases known to regulate mitosis.[82] Because of their role in cell cycle progression and the fact that they are overexpressed in leukemias and solid tumors,[83] aurora kinases make attractive targets in CML therapeutic development. Several compounds with activity against ABL mutants, including T315I, were developed and have entered clinical trials. Among these, the most tested candidate is AT9283 (Astex Therapeutics) with activity against ABL, as well as Aurora A/B kinases, and Janus kinases 2/3 (JAK2 and JAK3).[84] Preclinical efficacy was demonstrated in mouse models leading to initiation of clinical trials.[84] Phase I and IIa clinical trials were completed in October 2010, and a recommended phase II dose was determined (NCT00522990). Danusertib, another Aurora kinase inhibitor, is currently in phase I studies in patients with refractory Ph+ leukemias.[85] Results have not yet been published. Two other Aurora kinase inhibitors with activity against T315I mutant ABL, MK-0457 and XL228, failed in clinical trials (NCT00464113) for various reasons, including toxicity.[86] The clinical efficacy of compounds inactive against T315I, but which inhibit other pathways (like the Src-family kinases) remains to be determined. Table 1 provides an overview of new compounds in development for Ph+ leukemias.

Allosteric/Non-ATP Competitive Inhibitors

DCC-2036 (Deciphera) is an inhibitor of BCR-ABL that forces a conformational change of ABL on drug binding. ABL can exist in either an active (type I) or inactive (type II) conformation based on phosphorylation status. Structure-based design of DCC-2036 elucidated a "switch-pocket" in ABL, inducing a stable and inactive state.[87] DCC-2036 inhibits ABL in a non-ATP competitive manner; it also inhibits Src, Lyn, Fgr, Hck, Flt3, and Tie2 but spares Kit. Based on efficacy in preclinical studies, a phase I trial has been initiated and is currently recruiting.

An allosteric, non-ATP competitive inhibitor of BCR-ABL is GNF-2 (Genomics Novartis Foundation), which was discovered during kinase activity screening.[88] GNF-2 is hypothesized to bind at the myristoyl binding cleft of BCR-ABL, distant from

TABLE 1. Drugs Developed for CML Therapy With Activity Against ABL-Kinase and Other Kinases Listed

Novel ABL Inhibitors				
Inhibitor	**Non-ABL Kinase Target(s)**	**T315I**	**Status**	**References**
DCC-2036	Src, Lyn, Fgr, Hck, Flt3, Tie2	Active	Phase I/II	NCT00827138
GNF compounds	ABL only	Active	Preclinical	*
ON012380	ABL only	Active	Preclinical	†
PPY-A	ABL only	Active	Preclinical	‡,§
SGX393	ABL only	Active	Preclinical	¶
XL228	Aurora A/B, FAK, Src	Active	Phase I—terminated	NCT00464113
MK-0457	Aurora A-C, Flt3	Active	Phase II—terminated	NCT00405054
AT9283	Aurora A/B, JAK2/3	Active	Phase I/II	NCT00522990
Danusertib	Aurora A-C, Ret, Trk-A, FGFR-1	Active	Phase II	NCT00335868
Ponatinib	Flt3, FGFR, VEGFR, c-kit, PDGFR	Active	Phase II	NCT01207440
Bafetenib	Lyn	NA	Phase I—development unlikely	NCT00352677
AP23464	Src family	Active	Preclinical	‖
Bosutinib	Src, TEC, STE20, CAMK2G	NA	Phase I/II/III	NCT00811070, NCT00261846
DSA compounds	Src	Active	Preclinical	**
PD166326	Src	NA	No trials or recent reports	††
Saracatinib	Src family	NA	Not in trials for CML	‡‡
HG-7-85-01	Src, PDGFR, VEGFR, Flt3, Ret, Tie2, Kit, DDR1, b-raf	Active	Preclinical	§§

PLoS One. 2011;6:e15929.

†*Proc Natl Acad Sci U S A*. 2005;102:1992–1997.

‡*Chem Biol Drug Des*. 2007;70:171–181.

§*Med Res Rev*. 2011;31:1–41.

¶*Proc Natl Acad Sci U S A*. 2008;105:5507–5512.

‖*Chem Biol Drug Des*. 2010;75:223–227.

***Cancer Res*. 2009;69:2384–2392.

††*Blood*. 2005;105:3995–4003.

‡‡*Expert Opin Investig Drugs*. 2010;19:931–945.

§§*Blood*. 2010;115:4206–4216.

NA indicates not active.

the active site of BCR-ABL. GNF-2 has exceptional specificity for BCR-ABL, does not inhibit c-Kit, PDGFR, or other kinases (63 tested), and is nontoxic toward non–BCR-ABL–expressing cells.[88] GNF-2 has been found to enhance imatinib activity against BCR-ABL, whereas a GNF-2 analog (21a-I) was found to synergize with dasatinib against the T315I mutant.[89] Other GNF analogues are in development,[90,91] but none are currently in clinical trials.

The Essential BCR Coiled Coil

Oligomerization of BCR-ABL through the coiled-coil domain (Fig. 1) is essential for oncogenicity,[92,93] making this region an attractive target for therapeutic development.[94] Non-small-molecule inhibitors targeting the BCR coiled-coil are exciting alternatives that disrupt BCR-ABL oligomerization and activation. We have recently reported the disruption of BCR-ABL via a rationally designed mutant coiled-coil peptide.[95] Such peptides may reduce the risk of acquired resistance due to the numerous contact points between the coiled-coil and the protein or because peptides are not typical substrates for drug efflux transporters whose overexpression may lead to resistance.[85] Delivery strategies for peptide therapeutics to the CML cell are a current focus of our laboratory.

Degrading BCR-ABL

A natural compound in vegetables, PEITC, was found to kill T315I harboring cells in culture and from patient samples.[96] PEITC induces oxidative stress in CML cells leading to degradation of BCR-ABL. Another degradation strategy involves a novel ubiquitin cycle inhibitor, WP1130, reported to rapidly induce ubiquitination of BCR-ABL resulting in protein relocation into aggresomes, rendering it inactive. Both imatinib-sensitive and -resistant CML cells initiated apoptosis in response to WP1130.[97]

Hsp90 (heat shock protein 90) inhibitors geldanamycin and 17-AAG were shown to induce degradation of BCR-ABL protein in vitro.[98,99] Mechanistically, after dissociation of Hsp-90 from client proteins, Bag1 (B-cell lymphoma-2 [Bcl-2]–associated athanogene-1) mediates BCR-ABL localization to the proteasome and stimulates its degradation via an E3-ligase–dependent mechanism.[100] However, clinical trials in CML were disappointing.

Immunotherapy

In addition to small molecules, immunologic targeting of BCR-ABL, rather than kinase inhibition, may be effective. Interferon may function by inducing cytotoxic T-cell responses against myeloid antigens.[101] A more specific approach is vaccines targeting the BCR-ABL junction.[102,103] Despite some encouraging results, the efficacy of this approach remains unproven in the absence of a prospective randomized trial. Antibodies to the BCR-ABL junction have also been produced.[104,105] Updates to these are smaller fragments of antibodies such as iDabs,[106] including those specific to BCR-ABL,[107] and small

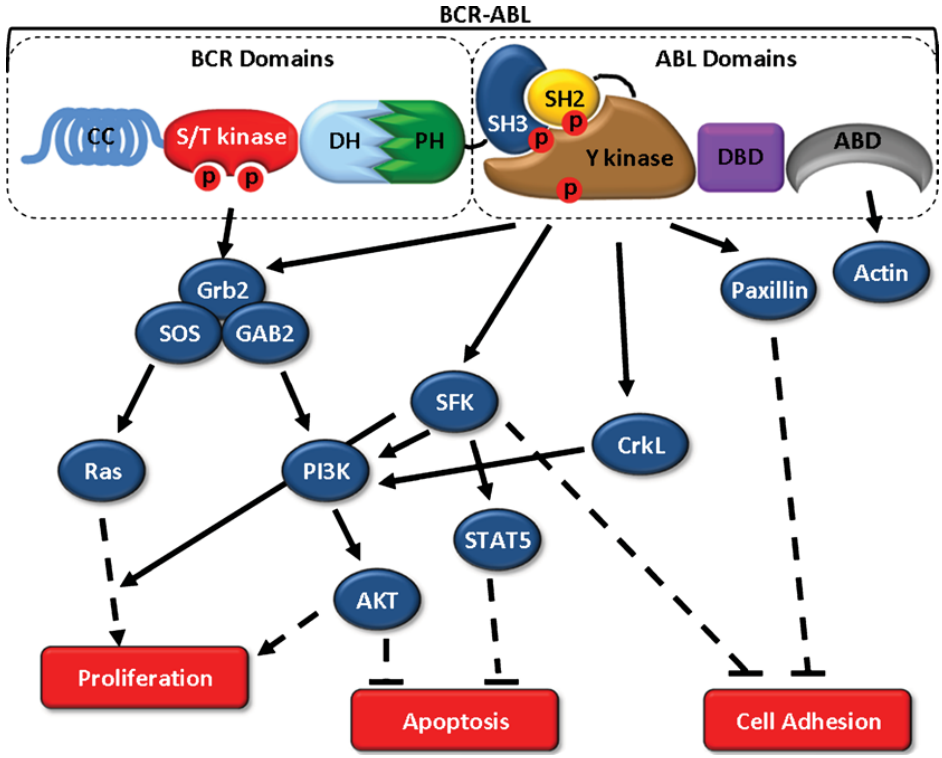

FIGURE 1. p210 BCR-ABL functional domains and effects of downstream signaling. BCR-ABL signaling leads to enhanced proliferation, reduced apoptotic potential, and altered cell adhesion. Contributions from both BCR and ABL domains on downstream signaling are illustrated. Dashed lines indicate additional intermediate signaling steps not detailed in this figure. ABD indicates actin binding domain; CC, coiled-coil; DBD, DNA binding domain; DH, Dbl homology; PH, Pleckstrin homology; S/T kinase, serine/threonine kinase; SH2 or SH3, Src homology 2/3; Y kinase, tyrosine kinase. Figure courtesy of Andrew Dixon.

antibody mimics or monobodies.[108] The clinical utility of these antibodies is unclear.

TARGETING CML STEM CELLS AND THEIR MICROENVIRONMENT

Stem Cell Niche

In vitro, TKIs are known to have antiproliferative effects on primitive CML cells, but they do not induce apoptosis. This may explain why TKIs fail to eliminate CML stem cells in vivo, evident by disease persistence and the inability to discontinue therapy. We have reported that primitive human CML stem cells are not dependent on BCR-ABL, suggesting that, on TKI challenge, CML stem cells rely on survival signals other than BCR-ABL. It is likely that these signals are provided by the microenvironment. It follows that therapies that only biochemically target BCR-ABL will be unable to eliminate CML stem cells.[71] Cytokines, chemokines, and the extracellular matrix, collectively referred to as the microenvironment, may activate signaling pathways involved in survival. Therapeutic strategies that target stem cells within this context hold promise to eliminate residual leukemia, including cytokine antagonists, adhesion molecule antagonists, and inhibitors of survival and self-renewal.[109]

The Hedgehog (Hh) signaling pathway has been implicated in hematopoietic stem cell renewal. Consistent with a critical role of Hh for CML pathogenesis, lack of Smoothened, an essential component of the pathway, was shown to attenuate CML in murine models.[110] Similarly, the Hedgehog inhibitor LDE225 in combination with nilotinib resulted in elimination of CML stem and progenitor cells.[111] Several Hedgehog inhibitors, including PF-04449913, for hematologic malignancies are also in clinical development.[112] Wnt/β-catenin signaling has also been shown to play a critical role in hematopoietic stem cell self-renewal and may offer therapeutic opportunities.[113]

AKT, a well-established downstream target of BCR-ABL, phosphorylates the Foxo3a transcription factor, leading to its exclusion from the nucleus and suppression of transcription. Despite this, Foxo3a is nuclear in primitive CML cells. Recent data have suggested that TGF-β signaling may be responsible for this unexpected finding, and it has been inferred that this may allow CML stem cells to remain in a quiescent state, despite BCR-ABL activity. If so, this would suggest that inhibiting TGF-β may push the critical cells into cycle, thereby rendering them susceptible to BCR-ABL inhibition. Efficient depletion of CML in vivo was found with a combination treatment using imatinib, a TGF-β inhibitor, and Foxo3a depletion.[114]

Yet another strategy is to interfere with stem cell homing. For example, CXCR4 is a receptor for the chemokine SDF-1 (stromal-derived factor 1), and plays a role in homing

TABLE 2. A Summary of Current Combination Therapies to Improve CML Treatment Outcomes in Clinical Trials

Combination Therapies for CML				
TKI	**Combination Second/Third Drug**	**Function of Non-TKI**	**Stage**	**Reference**
Any TKI	Arsenic trioxide	Multiple*	Phase I	NCT01397734
BOS/DAS	PF-04449913	Hh inhibitor	Phase I	NCT00953758
DAS	BMS-833923	smo inhibitor	Phase I/II	NCT01218477
DAS	Vorinostat	HDAC inhibitor	Phase I	NCT00816283
IM	Cytarabine or IFN	DNA synthesis or multiple†	Phase III	NCT00219739
IM	IFN	Multiple†	Phase II/IV	NCT00573378, NCT00390897
IM	IFN/granulocyte macrophage colony stimulating factor (GM-CSF)	Multiple†/GM differentiation	Unknown	NCT00050531
IM	Valproic acid	HDAC inhibitor	Phase II	NCT01011998
IM	Homoharringtonine (HHT)	Protein synthesis inhibitor	Phase II	NCT00114959
IM	Vatalanib (PTK 787)	VEGF, c-KIT, PDGFR inhibitor	Phase I/II	NCT00088231
IM	Zileuton	Alox5 inhibitor	Phase I	NCT01130688
IM	NIL	BCR-ABL	Phase II	NCT00769327
IM	Arsenic trioxide	Multiple*	Phase II	NCT00250042
IM	Lonafarnib	Farnesyl-OH-transferase inhibitor	Phase I	NCT00047502
IM	Tipifarnib	Farnesyltransferase inhibitor	Phase I	NCT00040105
IM	Vincristine/dexamethasone	Microtubule inhibitor/immunosuppressant	Phase II	NCT00763763
IM	GM-K562 - biologic	Immune surveillance initiation	Phase II	NCT00363649
IM	Everolimus	mTOR inhibitor	Phase I/II	NCT00093639
IM	Hydroxychloroquine	Lysosomal acidification/autophagy inhibitor	Phase II	NCT01227135
IM	TALL-104 - biologic	Modified therapeutic T cell	Phase II	NCT00415909
NIL	IFN	Multiple†	Phase I/II	NCT01220648, NTC01294618

*Proapoptotic/antiproliferative.

†Inhibits angiogenesis migration and proliferation.

BOS indicates bosutinib; DAS, dasatinib; GM, granulocyte and macrophage; HDAC, histone deacetylase; Hh, hedgehog; IM, imatinib; mTOR, molecular target of rapamycin; NIL, nilotinib; PDGFR, platelet-derived growth factor receptor; smo, smoothened; TKI, tyrosine kinase inhibitor; VEGF, vascular endothelial growth factor.

of CD34+ stem cells to the bone marrow microenvironment. Imatinib inhibition of BCR-ABL restores the CXCR4 interaction with SDF-1, leading to the migration and attachment of CML cells to the bone marrow microenvironment. However, a CXCR4 antagonist, AMD3465, partially inhibited cell migration to mesenchymal cells in coculture conditions. Similar results were seen with QLT0267, an integrin signaling inhibitor.[115]

Drug Combinations and Synthetic Lethality

Although stem cells express, but are not addicted to, BCR-ABL it may still be possible to manipulate other pathways which assume an essential role in response to ABL inhibition. This idea of synthetic lethality for cancer therapy is not new but has recently received more attention in the CML field propelled by emerging data demonstrating BCR-ABL–independent disease persistence on TKI therapy. In an RNAi-based screen for dysregulated genes in response to imatinib therapy, the Wnt pathway emerged as the viable target for a second hit.[116] Other critical pathways involved in disease progression or leukemic cell function have become attractive targets to augment BCR-ABL inhibition. For example, inhibition of ATG7,[117] MUC1,[118] Alox5,[119] and mTOR[120] have all been investigated in preclinical studies because they do not cause loss of hematopoietic stem cell function but instead target the leukemic clone in combination with TKIs. A list of recent clinical trials for combination therapies can be found in Table 2.

Finally, transcription factors such as STAT5 can mediate resistance to TKIs.[121] Some patients in BC-CML have significant down-regulation of signal transducers and activators of transcription (STAT) inhibitor proteins, potentiating cell survival and residual disease.[122] A new STAT5 inhibitor, pimozide, is able to decrease STAT5 and its target genes, resulting in growth inhibition of Ph+ patient samples independently of ABL mutations.[123] The precise mechanism of action of this compound is not known. For a comprehensive discussion on other signal transduction pathways in CML, the reader is referred to the referenced chapter.[124]

CONCLUSIONS

The rational design of drugs targeting BCR-ABL has made CML a manageable disease, resulting in prolonged survival for most patients. Mutations resulting in resistance to imatinib have driven development of the second-generation TKIs nilotinib and dasatinib. These inhibitors are active against a broad spectrum of BCR-ABL mutants, with the notable exception of the T315I "gatekeeper" mutant, which, in turn, has led to third-generation inhibitors. The most advanced of these is ponatinib, which has been termed a *pan-BCR-ABL inhibitor*, as it does not have identifiable gaps in BCR-ABL coverage. As complete ablation of BCR-ABL activity becomes a reality, the question arises whether we will see BCR-ABL–independent resistance emerge as a unifying feature of TKI failure. As the field has focused on the role of kinase domain mutations, relatively little is known about these mechanisms.

On the other side of the response spectrum is minimal residual leukemia despite prolonged TKI therapy. Although the relapse rate in this population of patients is very low, the need for continued treatment has major health and economic implications, and it remains possible that we will see unexpected late adverse effects in patients after decades of TKI therapy. Recent evidence suggests that primitive CML cells survive despite inhibition of BCR-ABL, suggesting a biologic barrier to disease eradication by TKIs.[71] We contend that eradicating CML will require targeting the stem cell niche. Several pathways have emerged as potential targets, and a clear winner has not yet been identified. In many respects, CML has served as a paradigm for cancer therapy, and it is likely that this will continue to be the case as we start to transform profound responses into definitive "cures."

REFERENCES

1. Kantarjian H, O'Brien S, Jabbour E, et al. Impact of treatment end point definitions on perceived differences in long-term outcome with tyrosine kinase inhibitor therapy in chronic myeloid leukemia. *J Clin Oncol*. 2011;29:3173–3178.
2. Signorovitch JE, Wu EQ, Betts KA, et al. Comparative efficacy of nilotinib and dasatinib in newly diagnosed chronic myeloid leukemia: a matching-adjusted indirect comparison of randomized trials. *Curr Med Res Opin*. 2011;27:1263–1271.
3. Tauchi T, Kizaki M, Okamoto S, et al. Seven-year follow-up of patients receiving imatinib for the treatment of newly diagnosed chronic myelogenous leukemia by the TARGET system. *Leuk Res*. 2011;35: 585–590.
4. Bartram CR, de Klein A, Hagemeijer A, et al. Translocation of c-*ab1* oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. *Nature*. 1983;306:277–280.
5. Rowley JD. Letter: a new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature*. 1973;243:290–293.
6. Anafi M, Gazit A, Gilon C, et al. Selective interactions of transforming and normal abl proteins with ATP, tyrosine–copolymer substrates, and tyrphostins. *J Biol Chem*. 1992;267:4518–4523.
7. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med*. 2001;344:1031–1037.
8. National Cancer Institute, National Institutes of Health. Surveillance Epidemiology and End Results Web site. Available at: http://seer.cancer.gov/statfacts/html/cmyl.html. Accessed July 14, 2011.
9. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. *Blood*. 2002;100: 2292–2302.
10. Wite LJ, Blackburn EK, Callender ST, et al. Chronic granulocytic leukaemia: comparison of radiotherapy and busulphan therapy. Report of the Medical Research Council's working party for therapeutic trials in leukaemia. *Br Med J*. 1968;1:201–208.
11. Hehlmann R, Heimpel H, Hasford J, et al. Randomized comparison of busulfan and hydroxyurea in chronic myelogenous leukemia: prolongation of survival by hydroxyurea. The German CML Study Group. *Blood*. 1993;82:398–407.
12. Rushing D, Goldman A, Gibbs G, et al. Hydroxyurea versus busulfan in the treatment of chronic myelogenous leukemia. *Am J Clin Oncol*. 1982;5:307–313.
13. Richards SM. Hydroxyurea versus busulphan for chronic myeloid leukaemia: an individual patient data meta-analysis of three randomized trials. Chronic myeloid leukemia trialists' collaborative group. *Br J Haematol*. 2000;110:573–576.
14. Pavlu J, Szydlo RM, Goldman JM, et al. Three decades of transplantation for chronic myeloid leukemia: what have we learned? *Blood*. 2011;117:755–763.
15. Breccia M, Palandri F, Iori AP, et al. Second-generation tyrosine kinase inhibitors before allogeneic stem cell transplantation in patients with chronic myeloid leukemia resistant to imatinib. *Leuk Res*. 2010;34: 143–147.
16. Oyekunle A, Klyuchnikov E, Ocheni S, et al. Challenges for allogeneic hematopoietic stem cell transplantation in chronic myeloid leukemia in the era of tyrosine kinase inhibitors. *Acta Haematol*. 2011;126:30–39.
17. Venepalli N, Rezvani K, Mielke S, et al. Role of allo-SCT for CML in 2010. *Bone Marrow Transplant*. 2010;45:1579–1586.
18. Talpaz M, Kantarjian HM, McCredie K, et al. Hematologic remission and cytogenetic improvement induced by recombinant human interferon alpha A in chronic myelogenous leukemia. *N Engl J Med*. 1986;314:1065–1069.

19. Dowding C, Gordon M, Guo AP, et al. Potential mechanisms of action of interferon-alpha in CML. *Leuk Lymphoma*. 1993;11(1 suppl):185–191.
20. Guilhot F, Lacotte-Thierry L. Interferon-alpha: mechanisms of action in chronic myelogenous leukemia in chronic phase. *Hematol Cell Ther*. 1998;40:237–239.
21. Ohnishi K, Ohno R, Tomonaga M, et al. A randomized trial comparing interferon-alpha with busulfan for newly diagnosed chronic myelogenous leukemia in chronic phase. *Blood*. 1995;86:906–916.
22. Tura S, Baccarani M, Zuffa E, et al. The Italian Cooperative Study Group on Chronic Myeloid Leukemia. Interferon alfa-2a as compared with conventional chemotherapy for the treatment of chronic myeloid leukemia. *N Engl J Med*. 1994;330:820–825.
23. Beck JR, Guilhot J, Giles FJ, et al. Cytarabine added to interferon improves the cost-effectiveness of initial therapy for patients with early chronic phase chronic myelogenous leukemia. *Leuk Lymphoma*. 2001;41:117–124.
24. Silver RT, Woolf SH, Hehlmann R, et al. An evidence-based analysis of the effect of busulfan, hydroxyurea, interferon, and allogeneic bone marrow transplantation in treating the chronic phase of chronic myeloid leukemia: developed for the American Society of Hematology. *Blood*. 1999;94:1517–1536.
25. Silberman G, Crosse MG, Peterson EA, et al. Availability and appropriateness of allogeneic bone marrow transplantation for chronic myeloid leukemia in 10 countries. *N Engl J Med*. 1994;331:1063–1067.
26. Kantarjian H, Shah NP, Hochhaus A, et al. Dasatinib versus imatinib in newly diagnosed chronic-phase chronic myeloid leukemia. *N Engl J Med*. 2010;362:2260–2270.
27. Nicolini FE, Turkina A, Shen ZX, et al. Expanding Nilotinib Access in Clinical Trials (ENACT): an open-label, multicenter study of oral nilotinib in adult patients with imatinib-resistant or imatinib-intolerant Philadelphia chromosome–positive chronic myeloid leukemia in the chronic phase. *Cancer*. 2011.
28. Saglio G, Kim DW, Issaragrisil S, et al. Nilotinib versus imatinib for newly diagnosed chronic myeloid leukemia. *N Engl J Med*. 2010;362:2251–2259.
29. Deininger M, O'Brien SG, Guilhot F, et al. International Randomized Study of Interferon Vs STI571 (IRIS) 8-year follow up: sustained survival and low risk for progression or events in patients with newly diagnosed chronic myeloid leukemia in chronic phase (CML-CP) treated with imatinib. *ASH Annual Meeting Abstracts*. 2009;114:1126.
30. Corm S, Micol J, Leroyer A, et al. Kinetic of chronic myeloid leukaemia (CML) prevalence in Northern France since the introduction of imatinib. *J Clin Oncol*. 2008;26(20 suppl):Abstract 7088.
31. Hantschel O, Rix U, Superti-Furga G. Target spectrum of the BCR-ABL inhibitors imatinib, nilotinib and dasatinib. *Leuk Lymphoma*. 2008;49:615–619.
32. Baccarani M, Castagnetti F, Gugliotta G, et al. Response definitions and European Leukemianet Management recommendations. *Best Pract Res Clin Haematol*. 2009;22:331–341.
33. Hughes TP, Kaeda J, Branford S, et al. Frequency of major molecular responses to imatinib or interferon alfa plus cytarabine in newly diagnosed chronic myeloid leukemia. *N Engl J Med*. 2003;349:1423–1432.
34. Radich JP. Measuring response to BCR-ABL inhibitors in chronic myeloid leukemia. *Cancer*. [published online ahead of print June 29, 2011] doi:10.1002/cncr.26280.
35. Druker BJ, Lydon NB. Lessons learned from the development of an abl tyrosine kinase inhibitor for chronic myelogenous leukemia. *J Clin Invest*. 2000;105:3–7.
36. Druker BJ, Tamura S, Buchdunger E, et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat Med*. 1996;2:561–566.
37. Talpaz M, Silver RT, Druker BJ, et al. Imatinib induces durable hematologic and cytogenetic responses in patients with accelerated phase chronic myeloid leukemia: results of a phase 2 study. *Blood*. 2002;99:1928–1937.
38. Sawyers CL, Hochhaus A, Feldman E, et al. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. *Blood*. 2002;99:3530–3539.
39. Kantarjian H, Sawyers C, Hochhaus A, et al. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. *N Engl J Med*. 2002;346:645–652.
40. O'Brien SG, Guilhot F, Larson RA, et al. Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. *N Engl J Med*. 2003;348:994–1004.
41. Druker BJ, Guilhot F, O'Brien SG, et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med*. 2006;355:2408–2417.
42. Cortes JE, Baccarani M, Guilhot F, et al. Phase III, randomized, open-label study of daily imatinib mesylate 400 mg versus 800 mg in patients with newly diagnosed, previously untreated chronic myeloid leukemia in chronic phase using molecular end points: tyrosine kinase inhibitor optimization and selectivity study. *J Clin Oncol*. 2010;28:424–430.
43. Baccarani M, Cortes J, Pane F, et al. Chronic myeloid leukemia: an update of concepts and management recommendations of European LeukemiaNet. *J Clin Oncol*. 2009;27:6041–6051.
44. Lombardo LJ, Lee FY, Chen P, et al. Discovery of *N*-(2-chloro-6-methylphenyl)-2-(6-(4-(2-hydroxyethyl)- piperazin-1-yl)-2-methylpyrimidin-4-ylamino)thiazole-5-carboxamide (BMS-354825), a dual Src/Abl kinase inhibitor with potent antitumor activity in preclinical assays. *J Med Chem*. 2004;47:6658–6661.
45. Lindauer M, Hochhaus A. Dasatinib. Recent results. *Cancer Res*. 2010;184:83–102.
46. Burgess MR, Skaggs BJ, Shah NP, et al. Comparative analysis of two clinically active BCR-ABL kinase inhibitors reveals the role of conformation-specific binding in resistance. *Proc Natl Acad Sci U S A*. 2005;102:3395–3400.
47. Talpaz M, Shah NP, Kantarjian H, et al. Dasatinib in imatinib-resistant Philadelphia chromosome–positive leukemias. *N Engl J Med*. 2006;354:2531–2541.
48. Hochhaus A, Kantarjian HM, Baccarani M, et al. Dasatinib induces notable hematologic and cytogenetic responses in chronic-phase chronic myeloid leukemia after failure of imatinib therapy. *Blood*. 2007;109:2303–2309.
49. Guilhot F, Apperley J, Kim DW, et al. Dasatinib induces significant hematologic and cytogenetic responses in patients with imatinib-resistant or -intolerant chronic myeloid leukemia in accelerated phase. *Blood*. 2007;109:4143–4150.
50. Cortes J, Rousselot P, Kim DW, et al. Dasatinib induces complete hematologic and cytogenetic responses in patients with imatinib-resistant or -intolerant chronic myeloid leukemia in blast crisis. *Blood*. 2007;109:3207–3213.
51. Shah NP, Kantarjian HM, Kim DW, et al. Intermittent target inhibition with dasatinib 100 mg once daily preserves efficacy and improves tolerability in imatinib-resistant and -intolerant chronic-phase chronic myeloid leukemia. *J Clin Oncol*. 2008;26:3204–3212.
52. Porkka K, Khoury HJ, Paquette RL, et al. Dasatinib 100 mg once daily minimizes the occurrence of pleural effusion in patients with chronic myeloid leukemia in chronic phase and efficacy is unaffected in patients who develop pleural effusion. *Cancer*. 2010;116:377–386.
53. Apperley JF, Cortes JE, Kim DW, et al. Dasatinib in the treatment of chronic myeloid leukemia in accelerated phase after imatinib failure: the START a trial. *J Clin Oncol*. 2009;27:3472–3479.
54. Weisberg E, Manley PW, Breitenstein W, et al. Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. *Cancer Cell*. 2005;7:129–141.
55. Weisberg E, Manley P, Mestan J, et al. AMN107 (nilotinib): a novel and selective inhibitor of BCR-ABL. *Br J Cancer*. 2006;94:1765–1769.
56. Kantarjian H, Giles F, Wunderle L, et al. Nilotinib in imatinib-resistant CML and Philadelphia chromosome–positive ALL. *N Engl J Med*. 2006;354:2542–2551.
57. le Coutre P, Ottmann OG, Giles F, et al. Nilotinib (formerly AMN107), a highly selective BCR-ABL tyrosine kinase inhibitor, is active in patients with imatinib-resistant or -intolerant accelerated-phase chronic myelogenous leukemia. *Blood*. 2008;111:1834–1839.

58. Kantarjian HM, Giles F, Gattermann N, et al. Nilotinib (formerly AMN107), a highly selective BCR-ABL tyrosine kinase inhibitor, is effective in patients with Philadelphia chromosome–positive chronic myelogenous leukemia in chronic phase following imatinib resistance and intolerance. *Blood*. 2007;110:3540–3546.
59. Koren-Michowitz M, le Coutre P, Duyster J, et al. Activity and tolerability of nilotinib: a retrospective multicenter analysis of chronic myeloid leukemia patients who are imatinib resistant or intolerant. *Cancer*. 2010;116:4564–4572.
60. Hochhaus A, Kreil S, Corbin AS, et al. Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy. *Leukemia*. 2002;16:2190–2196.
61. Gromicho M, Dinis J, Magalhaes M, et al. Development of imatinib and dasatinib resistance: dynamics of expression of drug transporters ABCB1, ABCC1, ABCG2, MVP, and SLC22A1. *Leuk Lymphoma*. 2011;52: 1980–1990.
62. Bradeen HA, Eide CA, O'Hare T, et al. Comparison of imatinib mesylate, dasatinib (BMS-354825), and nilotinib (AMN107) in an *N*-ethyl-*N*-nitrosourea (ENU)–based mutagenesis screen: high efficacy of drug combinations. *Blood*. 2006;108:2332–2338.
63. von Bubnoff N, Manley PW, Mestan J, et al. Bcr-Abl resistance screening predicts a limited spectrum of point mutations to be associated with clinical resistance to the Abl kinase inhibitor nilotinib (AMN107). *Blood*. 2006;108:1328–1333.
64. O'Hare T, Eide CA, Deininger MW. Bcr-Abl kinase domain mutations, drug resistance, and the road to a cure for chronic myeloid leukemia. *Blood*. 2007;110:2242–2249.
65. Laneuville P, Dilea C, Yin OQ, et al. Comparative in vitro cellular data alone are insufficient to predict clinical responses and guide the choice of BCR-ABL inhibitor for treating imatinib-resistant chronic myeloid leukemia. *J Clin Oncol*. 2010;28:e169–e171; author reply e72.
66. Branford S, Fletcher L, Cross NC, et al. Desirable performance characteristics for BCR-ABL measurement on an international reporting scale to allow consistent interpretation of individual patient response and comparison of response rates between clinical trials. *Blood*. 2008;112:3330–3338.
67. Hughes T, Saglio G, Branford S, et al. Impact of baseline BCR-ABL mutations on response to nilotinib in patients with chronic myeloid leukemia in chronic phase. *J Clin Oncol*. 2009;27:4204–4210.
68. Burke AC, Swords RT, Kelly K, et al. Current status of agents active against the T315I chronic myeloid leukemia phenotype. *Expert Opin Emerg Drugs*. 2011;16:85–103.
69. Mahon FX, Rea D, Guilhot J, et al. Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: the prospective, multicentre Stop Imatinib (STIM) trial. *Lancet Oncol*. 2010;11:1029–1035.
70. Deininger M. Hematology: curing CML with imatinib—a dream come true? *Nat Rev Clin Oncol*. 2011;8:127–128.
71. Corbin AS, Agarwal A, Loriaux M, et al. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *J Clin Invest*. 2011;121:396–409.
72. Giles FJ, Rosti G, Beris P, et al. Nilotinib is superior to imatinib as first-line therapy of chronic myeloid leukemia: the ENESTnd study. *Expert Rev Hematol*. 2010;3:665–673.
73. Mauro MJ, Deininger MW. Management of drug toxicities in chronic myeloid leukaemia. *Best Pract Res Clin Haematol*. 2009;22:409–429.
74. Gambacorti-Passerini C, Antolini L, Mahon FX, et al. Multicenter independent assessment of outcomes in chronic myeloid leukemia patients treated with imatinib. *J Natl Cancer Inst*. 2011;103:553–561.
75. Boschelli DH, Ye F, Wang YD, et al. Optimization of 4-phenylamino-3-quinolinecarbonitriles as potent inhibitors of Src kinase activity. *J Med Chem*. 2001;44:3965–3977.
76. Puttini M, Coluccia AM, Boschelli F, et al. In vitro and in vivo activity of SKI-606, a novel Src-Abl inhibitor, against imatinib-resistant Bcr-Abl+ neoplastic cells. *Cancer Res*. 2006;66:11314–11322.
77. Redaelli S, Piazza R, Rostagno R, et al. Activity of bosutinib, dasatinib, and nilotinib against 18 imatinib-resistant BCR/ABL mutants. *J Clin Oncol*. 2009;27:469–471.
78. Huang WS, Metcalf CA, Sundaramoorthi R, et al. Discovery of 3-[2-(imidazo[1,2-b]pyridazin-3-yl)ethynyl]-4-methyl-*N*-{4-[(4-methylpiperazin-1-yl)methyl]-3-(trifluoromethyl)phenyl}benzamide (AP24534), a potent, orally active pan-inhibitor of breakpoint cluster region-abelson (BCR-ABL) kinase including the T315I gatekeeper mutant. *J Med Chem*. 2010;53:4701–4719.
79. O'Hare T, Shakespeare WC, Zhu X, et al. AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance. *Cancer Cell*. 2009; 16:401–412.
80. Gozgit JM, Wong MJ, Wardwell S, et al. Potent activity of ponatinib (AP24534) in models of FLT3-driven acute myeloid leukemia and other hematologic malignancies. *Mol Cancer Ther*. 2011;10:1028–1035.
81. Cortes J, Talpaz M, Bixby D, et al. A phase 1 trial of oral ponatinib (AP24534) in patients with refractory chronic myelogenous leukemia (CML) and other hematologic malignancies: emerging safety and clinical response findings. *ASH Annual Meeting Abstracts*. 2010;116:210.
82. Carmena M, Earnshaw WC. The cellular geography of aurora kinases. *Nat Rev Mol Cell Biol*. 2003;4:842–854.
83. Howard S, Berdini V, Boulstridge JA, et al. Fragment-based discovery of the pyrazol-4-yl urea (AT9283), a multitargeted kinase inhibitor with potent aurora kinase activity. *J Med Chem*. 2009;52:379–388.
84. Tanaka R, Squires MS, Kimura S, et al. Activity of the multitargeted kinase inhibitor, AT9283, in imatinib-resistant BCR-ABL–positive leukemic cells. *Blood*. 2010;116:2089–2095.
85. Balabanov S, Gontarewicz A, Keller G, et al. Abcg2 overexpression represents a novel mechanism for acquired resistance to the multikinase inhibitor Danusertib in BCR-ABL–positive cells in vitro. *PLoS One*. 2011;6:e19164.
86. Vertex's Collaborator Merck Suspends Patient Enrollment in Clinical Trials of MK-0457 (VX-680) Pending Full Analysis of Clinical Data. 2007. Available at: www.drugs.com/clinical_trials/vertex-s-collaborator-merck-suspends-patient-enrollment-clinical-trials-mk-0457-vx-680-pending-full-2764.html. Accessed July 31, 2011.
87. Chan WW, Wise SC, Kaufman MD, et al. Conformational control inhibition of the BCR-ABL1 tyrosine kinase, including the gatekeeper T315I mutant, by the switch-control inhibitor DCC-2036. *Cancer Cell*. 2011;19:556–568.
88. Adrian FJ, Ding Q, Sim T, et al. Allosteric inhibitors of Bcr-Abl–dependent cell proliferation. *Nat Chem Biol*. 2006;2:95–102.
89. Deng X, Okram B, Ding Q, et al. Expanding the diversity of allosteric bcr-abl inhibitors. *J Med Chem*. 2010;53:6934–6946.
90. Zhang J, Adrian FJ, Jahnke W, et al. Targeting Bcr-Abl by combining allosteric with ATP-binding-site inhibitors. *Nature*. 2010;463:501–506.
91. Iacob RE, Zhang J, Gray NS, et al. Allosteric interactions between the myristate- and ATP-site of the Abl kinase. *PLoS One*. 2011;6:e15929.
92. McWhirter JR, Galasso DL, Wang JY. A coiled-coil oligomerization domain of Bcr is essential for the transforming function of Bcr-Abl oncoproteins. *Mol Cell Biol*. 1993;13:7587–7595.
93. Zhao X, Ghaffari S, Lodish H, et al. Structure of the Bcr-Abl oncoprotein oligomerization domain. *Nat Struct Biol*. 2002;9:117–120.
94. Beissert T, Puccetti E, Bianchini A, et al. Targeting of the N-terminal coiled coil oligomerization interface of BCR interferes with the transformation potential of BCR-ABL and increases sensitivity to STI571. *Blood*. 2003;102:2985–2993.
95. Dixon AS, Pendley SS, Bruno BJ, et al. Disruption of Bcr-Abl coiled coil oligomerization by design. *J Biol Chem*. 2011;286:27751–27760.
96. Zhang H, Trachootham D, Lu W, et al. Effective killing of Gleevec-resistant CML cells with T315I mutation by a natural compound PEITC through redox-mediated mechanism. *Leukemia*. 2008;22:1191–1199.
97. Sun H, Kapuria V, Peterson LF, et al. Bcr-Abl ubiquitination and Usp9x inhibition block kinase signaling and promote CML cell apoptosis. *Blood*. 2011;117:3151–3162.
98. Gorre ME, Ellwood-Yen K, Chiosis G, et al. BCR-ABL point mutants isolated from patients with imatinib mesylate-resistant chronic myeloid leukemia remain sensitive to inhibitors of the BCR-ABL chaperone heat shock protein 90. *Blood*. 2002;100:3041–3044.

99. Isaacs JS, Xu W, Neckers L. Heat shock protein 90 as a molecular target for cancer therapeutics. *Cancer Cell*. 2003;3:213–217.
100. Tsukahara F, Maru Y. Bag1 directly routes immature BCR-ABL for proteasomal degradation. *Blood*. 2010;116:3582–3592.
101. Burchert A, Wolfl S, Schmidt M, et al. Interferon-alpha, but not the ABL-kinase inhibitor imatinib (STI571), induces expression of myeloblastin and a specific T-cell response in chronic myeloid leukemia. *Blood*. 2003;101:259–264.
102. Bocchia M, Gentili S, Abruzzese E, et al. Effect of a p210 multipeptide vaccine associated with imatinib or interferon in patients with chronic myeloid leukaemia and persistent residual disease: a multicentre observational trial. *Lancet*. 2005;365:657–662.
103. Cathcart K, Pinilla-Ibarz J, Korontsvit T, et al. A multivalent bcr-abl fusion peptide vaccination trial in patients with chronic myeloid leukemia. *Blood*. 2004;103:1037–1042.
104. Telegeev GD, Dubrovska AN, Nadgorna VA, et al. Immunocytochemical study of Bcr and Bcr-Abl localization in K562 cells. *Exp Oncol*. 2010;32:81–83.
105. van Denderen J, Hermans A, Meeuwsen T, et al. Antibody recognition of the tumor-specific Bcr-Abl joining region in chronic myeloid leukemia. *J Exp Med*. 1989;169:87–98.
106. Perez-Martinez D, Tanaka T, Rabbitts TH. Intracellular antibodies and cancer: new technologies offer therapeutic opportunities. *Bioessays*. 2010;32:589–598.
107. Tse E, Lobato MN, Forster A, et al. Intracellular antibody capture technology: application to selection of intracellular antibodies recognising the BCR-ABL oncogenic protein. *J Mol Biol*. 2002;317:85–94.
108. Wojcik J, Hantschel O, Grebien F, et al. A potent and highly specific FN3 monobody inhibitor of the Abl SH2 domain. *Nat Struct Mol Biol*. 2010;17:519–527.
109. Konopleva MY, Jordan CT. Leukemia stem cells and microenvironment: biology and therapeutic targeting. *J Clin Oncol*. 2011;29:591–599.
110. Zhao C, Chen A, Jamieson CH, et al. Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. *Nature*. 2009;458:776–779.
111. Irvine DA, Zhang B, Allan EK, et al. Combination of the hedgehog pathway inhibitor LDE225 and nilotinib eliminates chronic myeloid leukemia stem and progenitor cells. American Society of Hematology Annual Meeting; December 5, 2009: New Orleans, LA.
112. Sheridan C. Genentech obtains proof of concept for hedgehog inhibition. *Nat Biotech*. 2009;27:968–969.
113. Zhao C, Blum J, Chen A, et al. Loss of beta-catenin impairs the renewal of normal and CML stem cells in vivo. *Cancer Cell*. 2007;12:528–541.
114. Naka K, Hoshii T, Muraguchi T, et al. TGF-beta–FOXO signalling maintains leukaemia-initiating cells in chronic myeloid leukaemia. *Nature*. 2010;463:676–680.
115. Jin L, Tabe Y, Konoplev S, et al. CXCR4 up-regulation by imatinib induces chronic myelogenous leukemia (CML) cell migration to bone marrow stroma and promotes survival of quiescent CML cells. *Mol Cancer Ther*. 2008;7:48–58.
116. Gregory MA, Phang TL, Neviani P, et al. Wnt/Ca^{2+}/NFAT signaling maintains survival of Ph+ leukemia cells upon inhibition of Bcr-Abl. *Cancer Cell*. 2010;18:74–87.
117. Bellodi C, Lidonnici MR, Hamilton A, et al. Targeting autophagy potentiates tyrosine kinase inhibitor–induced cell death in Philadelphia chromosome–positive cells, including primary CML stem cells. *J Clin Invest*. 2009;119:1109–1123.
118. Brossart P, Schneider A, Dill P, et al. The epithelial tumor antigen MUC1 is expressed in hematological malignancies and is recognized by MUC1-specific cytotoxic T-lymphocytes. *Cancer Res*. 2001;61:6846–6850.
119. Chen Y, Hu Y, Zhang H, et al. Loss of the *Alox5* gene impairs leukemia stem cells and prevents chronic myeloid leukemia. *Nat Genet*. 2009;41:783–792.
120. Mancini M, Corradi V, Petta S, et al. mTOR inhibitor RAD001 (Everolimus) enhances the effects of imatinib in chronic myeloid leukemia by raising the nuclear expression of c-ABL protein. *Leuk Res*. 2010;34:641–648.
121. Warsch W, Kollmann K, Eckelhart E, et al. High STAT5 levels mediate imatinib resistance and indicate disease progression in chronic myeloid leukemia. *Blood*. 2011;117:3409–3420.
122. Melo JV, Barnes DJ. Chronic myeloid leukaemia as a model of disease evolution in human cancer. *Nat Rev Cancer*. 2007;7:441–453.
123. Nelson EA, Walker SR, Weisberg E, et al. The STAT5 inhibitor pimozide decreases survival of chronic myelogenous leukemia cells resistant to kinase inhibitors. *Blood*. 2011;117:3421–3429.
124. Melo JV, Goldman JM, Deininger M. Signal transduction inhibitors in chronic myeloid leukemia. In: *Myeloproliferative Disorders*. Berlin, Germany: Springer; 2007:75–102.

CHAPTER 3

DISRUPTING BCR-ABL IN COMBINATION WITH SECONDARY LEUKEMIA-SPECIFIC PATHWAYS IN CML CELLS LEADS TO ENHANCED APOPTOSIS AND DECREASED PROLIFERATION

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Disrupting BCR-ABL in Combination with Secondary Leukemia-Specific Pathways in CML Cells Leads to Enhanced Apoptosis and Decreased Proliferation

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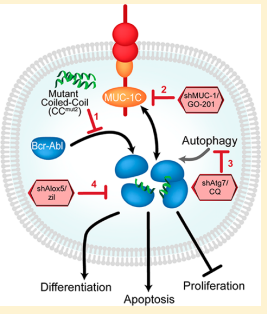
Supporting Information

ABSTRACT: Chronic myeloid leukemia (CML) is a myeloproliferative disorder caused by expression of the fusion gene BCR-ABL following a chromosomal translocation in the hematopoietic stem cell. Therapeutic management of CML uses tyrosine kinase inhibitors (TKIs), which block ABL-signaling and effectively kill peripheral cells with BCR-ABL. However, TKIs are not curative, and chronic use is required in order to treat CML. The primary failure for TKIs is through the development of a resistant population due to mutations in the TKI binding regions. This led us to develop the mutant coiled-coil, CC^{mut2}, an alternative method for BCR-ABL signaling inhibition by targeting the N-terminal oligomerization domain of BCR, necessary for ABL activation. In this article, we explore additional pathways that are important for leukemic stem cell survival in K562 cells. Using a candidate-based approach, we test the combination of CC^{mut2} and inhibitors of unique secondary pathways in leukemic cells. Transformative potential was reduced following silencing of the leukemic stem cell factor Alox5 by RNA interference. Furthermore, blockade of the oncogenic protein MUC-1 by the novel peptide GO-201 yielded reductions in proliferation and increased cell death. Finally, we found that inhibiting macroautophagy using chloroquine in addition to blocking BCR-ABL signaling with the CC^{mut2} was most effective in limiting cell survival and proliferation. This study has elucidated possible combination therapies for CML using novel blockade of BCR-ABL and secondary leukemia-specific pathways.



KEYWORDS: *CML, coiled-coil, CC^{mut2}, zileuton, GO-210, chloroquine, combination therapy, K562, BCR-ABL*

INTRODUCTION

Chronic myeloid leukemia (CML) manifests following a reciprocal chromosomal translocation between the breakpoint cluster region (BCR) gene and the Abelson tyrosine kinase (ABL) gene [t(9;22)(q34;q11)] in the hematopoietic stem cell.[1,8] Upon expression of the BCR-ABL fusion protein (a constitutively active tyrosine kinase), a leukemic stem cell (LSC) is generated, driving LSC self-renewal and expansion of BCR-ABL-expressing lineages including myeloid and lymphoid blasts.[9,10] Tyrosine kinase inhibitors (TKIs) are competitive inhibitors for the ATP binding site of ABL and make up the therapeutic arsenal for disease management.[3] We have previously described a unique interfering peptide, CC^{mut2}, able to disrupt BCR-ABL homo-oligomers.[4] Moreover, oligomerization is necessary for ABL activation.[11] Interestingly, oligomeric disruption of trans-auto phosphorylation by CC^{mut2} exacts its activity via the coiled-coil domain in BCR, leading to an overall similar effect seen with TKIs: reduced phosphorylation of ABL and downstream targets STAT5 and Crk-L, induction of apoptosis, and reduction in proliferation.[4,12]

Single-agent TKI therapy for CML has effectively limited disease progression for the majority of patients.[3] However, resistance to therapy and persistence of a subset of leukemic cells despite TKI activity[13] demonstrate the necessity for multiple-agent therapy, especially to address the LSC population.[10,14] Previous reports have demonstrated enhanced cytotoxicity when using a TKI in combination with a second agent targeting a BCR-ABL independent pathway,[6,7,15,16] two of which have moved into clinical trials (NCT01130688; NCT01227135). Though these are promising developments to circumvent molecular failure, the TKI component will likely continue to have problems with resistance.[2,17,18] The CC^{mut2} may be less prone to mutational resistance selection, mainly due to the highly specific and selective nature of a large interaction domain.[4] This draws a parallel similar to differences in specificity between small molecules versus antibodies for cancer therapy.[19] Therefore, a multiple-agent therapeutic approach involving the CC^{mut2} may be superior to TKI single-agent therapy (Figure 1A).

We were interested in discovering whether enhanced apoptotic activation or reduction in proliferation could be achieved by combining CC^{mut2} with secondary agents having independent mechanisms of action.[20] Here, we detail the results

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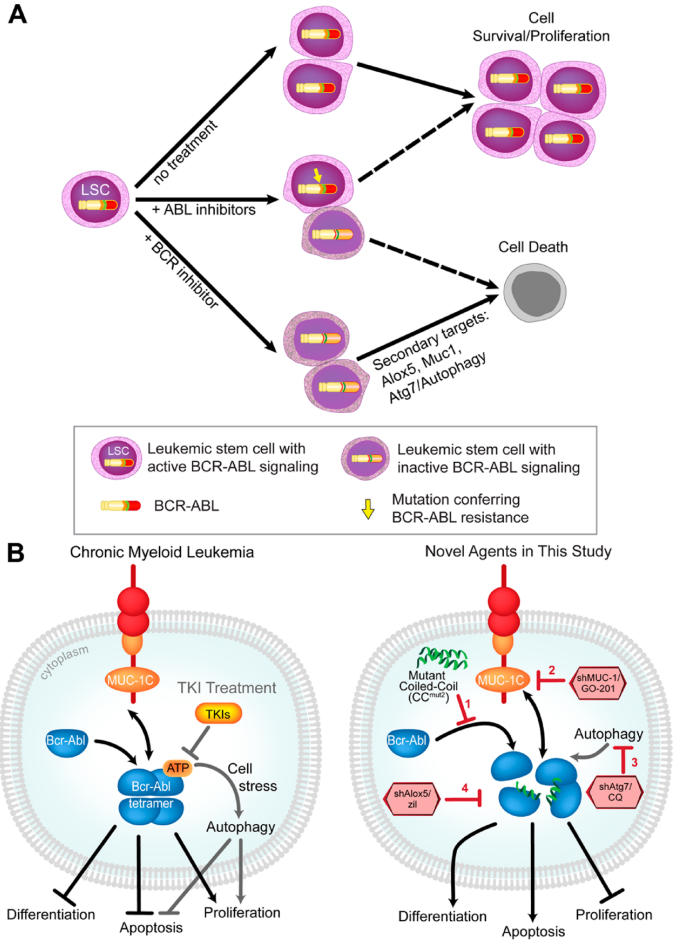

Figure 1. (A) Model of multiagent vs single agent therapy for CML. The BCR-ABL translocation is thought to arise in the stem cell population, giving rise to leukemic stem cells (LSC). If untreated, the progeny from the LSC expressing the constitutively active tyrosine kinase fusion protein BCR-ABL give rise to expanded cell populations, which is fatal to the patient. Current clinical care uses targeted therapy against the kinase ABL, which does treat the disease. However, mutations within the ABL gene can generate a resistant population, which can lead to leukemic cell survival. Using a BCR-targeted inhibitor is hypothesized to decrease the possibility of resistance, and adding a second agent can enhance cell killing. (B) Left: a CML cell and a simplified disease pathway of CML (black arrows). Current treatment modalities (TKIs) can act to reverse these effects; however, a potential escape from conventional therapy through the autophagy pathway is depicted with gray arrows. Right: experimental strategies for addressing untreated or resistant populations of CML disease states. (1) The CC^{mut2} targets the BCR domain with an effect on *in vitro* cells similar to that of TKIs; (2) the oncoprotein MUC-1C can be disrupted by RNA interference (RNAi) or the peptide inhibitor GO-201; (3) autophagy blockade can be achieved by chloroquine (CQ) or by RNAi targeting Atg7; (4) the LSC enhancing factor Alox5 is inhibited by targeting 5-LO metabolism with zileuton (zil) or degrading the Alox5 message using RNAi.

of this peptide (delivered as a gene and transcribed *in vitro*) in combination with additional small molecule or biologic agents. Secondary target candidates were selected based on previous reports of drugs known to be effective when used in combination with imatinib, and those drugs were found to be effective only against leukemic (vs normal hematopoietic) cells.[18] Additional criteria led to the selection of key proteins involved in CML progression, namely, ATG7,[7] MUC-1,[21] and Alox5.[15] These targets were chosen because they do not cause loss of hematopoietic stem cell function. RNA interference (RNAi) or molecular disruption of these pathways was investigated.

The selection of and rationale for pathways and molecular agents for combination with the CC^{mut2} are as follows: (1) ATG7 and chloroquine (CQ). Macroautophagy (referred to as autophagy from this point on) is a cellular process activated in starvation conditions to improve recycling of cell components and enhance cell survival.[22] This pathway becomes important in cancer as a mechanism for cell escape elimination when treated with anticancer agents.[23] ATG7 is necessary for autophagy, and its inhibition blocks the formation of the autophagosome, an early step in the autophagy process, while CQ inhibits lysosomal acidification, which eliminates the breakdown of products contained in the autophagosome blocking cellular autophagy in the later stages.[23] Specifically in CML, a

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combination of imatinib and CQ enhance leukemic cell killing.[7] For this reason, we were interested in blocking autophagy in combination with CC^{mut2}. (2) Alox5 and zileuton (zil). The arachidonate 5-lipoxygenase (Alox5) gene product 5-lipoxygenase (5-LO) is responsible for leukotriene synthesis from arachadonic acid (AA); reports indicate increased AA in CML cells.[5,18] This enhances proliferation and inhibits differentiation in leukemic stem cells (with Bcr-Abl). Knockout of Alox5 or treatment with a 5-LO inhibitor (zil) is known to change the proliferative capacity of CML cells.[15,24,25] (3) MUC-1C and GO-201. MUC-1 is a known oncogene widely expressed in cancer cells in general and in CML cells specifically.[21,26] Kufe and colleagues have reported the association of the cytoplasmic portion of MUC-1 (MUC-1C) with BCR-ABL, enhancing the oncogenic cytoplasmic signaling of BCR-ABL. Furthermore, the use of GO-201, a specific inhibitor of MUC-1C with imatinib, has shown reduction in proliferation and induced differentiation in CML cells.[6,16] These interactions are depicted in Figure 1B.

In this article, these agents in combination with CC^{mut2} were found to improve therapeutic potency in K562 cells. Transformative ability was most reduced by inhibiting protein expression of Alox5 using RNAi in combination with the CC^{mut2}. Reduction in proliferative capacity resulted largely due to CC^{mut2} alone but was further decreased by GO-201. Finally, increased caspase activity was seen with CQ and CC^{mut2}, while combinations of either GO-201 or CQ and CC^{mut2} enhanced the apoptotic and necrotic cell population as visualized by Annexin-V and 7-AAD staining.

MATERIALS AND METHODS

Constructs. RNAi constructs were targeted against human Atg7, Alox5, MUC-1, or luciferase control. Target sequences for Atg7 or MUC-1 were derived from previous reports,[6,7] while the Alox5 (NM_000698) RNAi sequence was designed using BLOCK-iT RNAi Designer (Life Technologies, Grand Island, NY). RNAi sequences are as follows: Alox5 (5′-ggaatgacttcgccgactttg-3′); Atg7 (5′-cagtggatctaaatctcaaactgat-3′);[7] MUC-1C (5′-aagttcagtgcccagctctac-3′).[6] Oligonucleotides encoding short hairpins against the following transcripts were synthesized at the University of Utah core facilities: Alox5 top (5′- gatccggaatgacttcgccgactttggaagcttgcaaagtcggcgaagtcattccttttttggaagc-3′) and bottom (5′- ggccgcttccaaaaaaggaatgacttcgccgactttgcaagcttccaaagtcggcgaagtcattccg-3′); Atg7 top (5′- gatccgcagtggatctaaatctcaaactgatgaagcttgatcagtttgagatttagatccactgttttttggaagc-3′) and bottom (5′-ggccgcttccaaaaaacagtggatctaaatctcaaactgatcaagcttcatcagtttgagatttagatccactgcg-3′); MUC-1 top (5′-gatccgaagttcagtgcccagctctacgaagcttggtagagctgggcactgaacttttttttggaagc-3′) and bottom (5′- ggccgcttccaaaaaaaagttcagtgcccagctctaccaagcttcgtagagctgggcactgaacttcg-3′). Top and bottom strands were annealed and then cloned into the Gene Silencer shRNA Expression Vector pGSH1-GFP (Genlantis, San Diego, CA) according to the manufacturer's instructions. The pEGFP-C1 parent plasmid was purchased from Clontech laboratories (Mountain View, CA); the coiled-coil (pEGFP-CC) or mutant coiled-coil (pEGFP-CC^{mut2}) are described elsewhere.[4]

Cell Lines and Transfections. Cells culture and transfections were carried out as described previously.[4] Briefly, K562 cells were cultured in complete RPMI medium with 10% FBS, 1% Pen-Strep-Glut, and 0.1% Gentamycin (Life Technologies, Grand Island, NY). Cells were maintained at 37 °C and 5% CO_2. DNA constructs were transfected with the Amaxa nucleofection system (Lonza Bio, Basel, Switzerland), using 6 μg of DNA in 100 μL of Solution V with 2 million cells, and then returned to complete RPMI medium.

Western Blotting. Cells were counted on a hemocytometer, pelleted, and frozen at −80 °C overnight. Cell pellets were resuspended in RIPA lysis and extraction buffer (Thermo Scientific/Pierce Protein Biology Products, #89900, Kalamazoo, MI). A BCA Protein assay was performed (Thermo Scientific, #23225), and 10 μg of protein was loaded in each lane of a denaturing gel. Standard Western blotting procedures were used.[4] Antibodies used to detect Atg7 (Sigma-Aldrich, #A2856, St. Louis, MO), LC3A/B (Sigma-Aldrich, #L8918), MUC-1C (Thermo Scientific, #MUC-1 Ab-5/HM-1630), Alox5 (Abcam, #ab115764, Cambridge, MA), actin (Abcam, #ab1801), and eIF4E (Cell Signaling Technology, #C46H6, Danvers, MA) primary antibodies were used. HRP conjugated secondary antibodies were anti-Armenian hamster (Abcam, #ab5745) or anti-rabbit (Cell Signaling Technology, #7074). Quantification of bands using relative densitometry was completed using AlphaView SA (Protein Simple, v3.0, Santa Clara, CA). For LC3II/I ratios, background corrected sum values for each band were calculated as a ratio to eIF4E control. Then LC3-II percent control was divided by LC3-I percent control. This gives a relative ratio of LC3-II to LC3-I conversion.

Colony Forming Assay. pGSH1 constructs expressing shRNA sequences against Atg7, MUC-1, or Alox5 were transfected and cultured 4 days to ensure knockdown. One day following transfection, genticin reagent (Life Technologies) was added at a concentration of 500 μg/mL in complete RPMI. On day 4, a second construct was transfected (pEGFP-C1; −CC; or $-CC^{mut2}$). Dual-transfected cells were resuspended in Iscove's Modified Dulbecco's Medium containing 2% FBS (Stem Cell Technologies, Vancouver, BC, Canada), and 1,000 cells were seeded in Methocult H4230 methylcellulose medium (Stem Cell Technologies). Imatinib mesylate (IM #CT-IM001, Chemie-Tek, Indianapolis, IN) was added to untransfected K562 cells in Methocult at the time of seeding. Transformation potential was assessed 7 days after seeding cells by counting colonies in 200 μM^2.

Drug Treatments. In all cases, small molecule or peptide-based inhibitors were added to transfected cells 6 h after transfections unless otherwise noted. GO-201 (Sigma-Aldrich, #G7923) is a well-described peptide inhibitor of MUC-1C[27] and was used at a final concentration of 5 μM in 1× PBS (Life Technologies, #14190-144). Zileuton (Sigma-Aldrich, #Z4277) is a small molecule inhibitor active against 5-lipoxygenase (the protein product of Alox5) and was used at a final concentration of 20 μM dissolved in DMSO. Chloroquine (Sigma-Aldrich, #C6628) is an inhibitor of lysosomal acidification (autophagosomal activation) and was used at a final concentration of 10 μM.

Cell Proliferation. K562 cells were transfected with EGFP, CC, or CC^{mut2}, followed by drug treatment 6 h later. Trypan blue exclusion[4] was assessed 48 h after transfection to determine cell proliferation/viability.

Caspase 3/7 Assay. Cells were transfected as indicated above (in Cell Lines and Transfections) followed by drug treatment 6 h later. Forty-eight hours following transfection, cells were counted, pelleted, and frozen at −80 °C overnight. Cells were resuspended, lysed, and processed according to the Caspase Glo 3/7 manufacturer's instructions (Promega, Madison, WI). Luminescence was measured after 1 h at 26

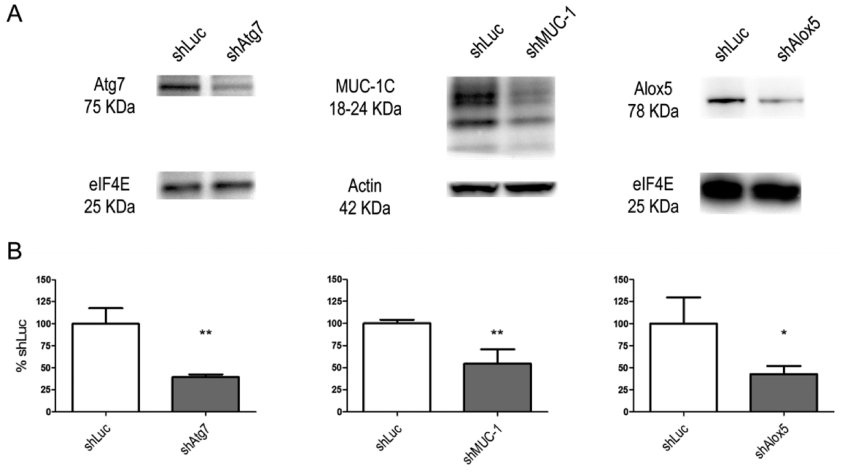

Figure 2. (A) Representative Western blot of protein expression for Atg7, MUC-1, and Alox5 after 96 h following transfection with pGSH1-shLUC-GFP (left lane) compared to RNA interference targeting genes of interest (right lane). (B) Knockdown via RNAi is depicted based on semiquantitative band densitometry for each construct compared to that of shLuc control. Each short-hairpin RNAi construct resulted in significant knockdown of its targets. Student's *t* test, $^{**}p < 0.01$; $^{*}p < 0.05$; $n = 3$.

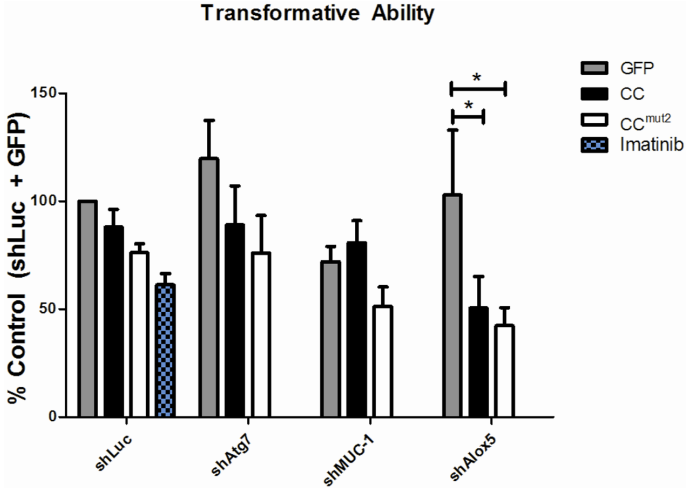

Figure 3. Colony forming assay of dual-transfected short-hairpin and experimental constructs reveals a significant combination effect of Alox5 and CC or CC^{mut2}. Two-way ANOVA and the Bonferroni post-test, $^{*}p < 0.05$, $n \geq 3$.

°C on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA).

Annexin-V/7-AAD Assay. Cells were transfected and treated as described above (in Cell Lines and Transfections) followed by drug treatment 6 h later. At 48, 72, or 96 h, cells were resuspended in 0.5 mL of Annexin-V binding buffer (Life Technologies, #V13246) stained with 1 nM 7-AAD (Life Technologies, # A1310) and a 1:20 dilution of Annexin-V-APC (Life Technologies, #A35110). Samples were sorted using a BD FACSCanto II flow cytometer according to GFP positivity (10,000 GFP events were collected). Cells were then sorted according to apoptotic (Annexin-V) and necrotic (7-AAD) markers. Data was further analyzed using FlowJo flow cytometry analysis software (Tree Star Inc., Ashland, OR).

Statistics. Data are expressed as the means ± SEM from at least 3 independent experiments. Significance of differences between groups was assessed in GraphPad Prism 5 (GraphPad Software, La Jolla, CA) using either a Student's *t* test, two-way ANOVA with a Bonferroni post-test, or one-way ANOVA with Bonferroni post-test. A *p*-value of <0.05 was considered significant.

RESULTS

BCR-Based Inhibition of BCR-ABL with Alox5 Knockdown Reduces the Transformation Potential of K562 Cells. 5-Lipoxygenase (5-LO), the protein product of Alox5, mediates processes such as inflammation and oxidative stress through leukotriene synthesis. Because of this, 5-LO antagonists are an important therapy for inflammatory diseases and

have also been suggested for cancer therapy.[28] Reports of potential antiproliferative effects in hematologic malignancies from the loss of Alox5 or 5-LO inhibitors surfaced in the 1980s[29,30] and recently were bolstered in a CML *in vivo* model by data from Chen and colleagues.[5,15,31]

To determine the contribution of several pathways to transformative ability (measured by colony forming cells), selected pathways were disrupted by knockdown of key genes regulating each pathway using shRNA expressing constructs. Western blotting for protein products of Atg7, MUC-1, and Alox5 demonstrated successful knock down of all targets (Figure 2A, second lane of each pair) when compared to that in control shRNA against luciferase control (Figure 2A, shLuc, first lane of each pair). These data are quantified using band densitometry and expressed as percent shLuc (Figure 2B). These constructs were then used in combination with GFP control, wild-type coiled-coil (CC), or mutant coiled-coil (CC^{mut2})[4] in a week long transformation study. Transient expression of shAlox5 and CC or CC^{mut2} resulted in significant reduction of colonies (Figure 3, shAlox5 group, far right bars) compared to that in GFP control and shAlox5 dual expression (Figure 3, shAlox5 group, third to right bar, gray).

Chloroquine Combinations Block an Upregulated Autophagy Pathway Following BCR-ABL Inhibition. Autophagy is a degradative process used by cells to break down intracellular material via lysosomes. Autophagy can promote or suppress oncogenesis depending on the cellular context;[22] however, induction of autophagy provides a survival mechanism in BCR-ABL cells treated with imatinib (cells undergoing stress; see Figure 1B).[7,32] Though previous reports indicated enhanced autophagy following the introduction of kinase inhibitors,[7,32,33] this pathway has not been previously investigated following the expression of the CC^{mut2}. Additionally, since no reduction in transformative ability was observed following the knockdown of Atg7, we proceeded to investigate whether autophagy is activated following the transfection of GFP, CC, or CC^{mut2}. The conversion of microtubule associated protein light chain 3 (LC3) from LC3-I to LC3-II was used to monitor autophagy. This is the case when comparing lanes 3 and 4 or lanes 7 and 8. Because cellular levels of LC3-II are indicative of the number of autophagosomes (e.g., an increase in autophagy),[34] increases in LC3-II protein expression were measured by immunobloting. When no autophagy occurs, a more prominent LC3-I band will be visible, indicating the presence of the precursor and cytoplasmic LC3-I (i.e., LC3II/I <1.0). This is visible in Figure 4, lanes 1−2 and 5−6. When autophagy occurs, and inhibitors are added to eliminate LC3-II breakdown via the lysosome, LC3-II becomes the prominent band (i.e., LC3II/I >1.0).[34,35]

LC3-II/LC3-I ratios[7,35] were calculated following exposure to 5 μM imatinib (Figure 4, lanes 3 and 4) or transfections and with or without CQ at 24 h (Figure 4, lanes 5−8). The data represented in Figure 4 demonstrate little to no activation of autophagy in untreated or GFP transfected K562 cells (Figure 4, lanes 1−2; 5−6). Autophagy is activated by inhibition of BCR-ABL using either BCR or ABL inhibitors at 24 h (Figure 4, lanes 3−4; 7−8). Similar trends were seen at 12 h (data not shown).

CQ and GO-201 Further Diminish the Proliferative Capacity of K562 Cells beyond the Reductions Seen Using CC^{mut2} Alone. MUC-1 is a membrane-bound glycosylated phosphoprotein whose normal function protects the body from chemicals/bacteria through its polarized

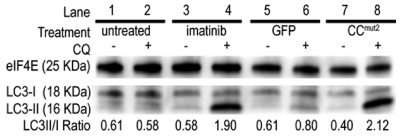

Figure 4. Autophagy occurs in K562 cells and is increased following exposure to IM or the mutant coiled-coil (CC^{mut2}). Shown is a representative Western blot for LC3-I/II and at 24 h. Treatment with imatinib or transfection CC^{mut2} in concert with CQ treatment increases autophagic flux, indicated by LC3-II expression compared to that of LC3-I. This is given as the LC3-II/I ratio at 24 h indicated in the table below the figure. n = 3.

expression on epithelial cell surfaces.[21] The cytoplasmic domain of MUC-1, called MUC-1C, interacts with the BCR portion of BCR-ABL to promote continued oncogenic signaling.[6] A peptide inhibitor of MUC-1C has been shown to downregulate BCR-ABL and inhibit cell growth,[16] indicating its value as a therapeutic target.

We previously demonstrated[4] decreased proliferation following the transient expression of CC^{mut2}, and these data are represented here (Figure 5, vehicle treated). To determine the effects of selected pathways in combination with BCR-mediated inhibition of ABL by the mutant coiled-coil, additional experiments were carried out as before[4] with drugs added 6 h after transfection. Untreated groups were scaled to previous data and compared to treated cells at 48 h. A significant reduction was seen across all samples when treated with the CC^{mut2} (Figure 5, white bars), consistent with previous work,[4] and further reductions were seen in the CQ and GO-201 treated groups when transfected with CC^{mut2} (CQ and GO-201 groups, white bars). In fact, GO-201 treatment reduces proliferation independent of the transfected group but further diminishes reduction in proliferation most in combination with the mutant coiled-coil (GO-201 group, white bars).

CQ Enhances the Activity of Effector Caspase 3/7 in Concert with CC^{mut2}. We next investigated whether increases in apoptotic signaling could be enhanced using the transient transfection of constructs with drug combinations at 48 h. Early apoptotic events can be indicated by executioner caspase 3 or 7 cleavage products. These products were measured using a luminescent DEVD substrate.[36] The only combination which resulted in a significant (3 fold) increase in caspase 3/7 activity was the CC^{mut2} with the CQ treated group (Figure 6; compare GFP and CC^{mut2} bars in the CQ group). Other combinations did not significantly enhance caspase 3/7 activity.

Increased Apoptotic and Necrotic Activity Are Observed Following CQ Combinations. In addition to effector caspase activity, later stage apoptosis and necrosis were measured using cell permeable reagents demonstrating apoptotic (phosphotidylserine externalization recognized by Annexin-V) or necrotic (nuclear membrane permeability by DNA intercalation of 7-AAD) events. At the earliest time measured, CQ and GO-201 increased cell death response (Figure 7A and D), though GO-201 affected only the CC^{mut2} transfected population, and was not significantly different from the vehicle treated group (Figure 7D). CQ has a broader impact potentiating the CC transfected population specifically (Figure 7A). Seventy-two hours after initial transfection, the singular impact of the CC^{mut2} transfected cells becomes clear as significant increases are seen in the apoptotic population (Figure 7B). The GO-201 apoptotic increase seen at 48 h

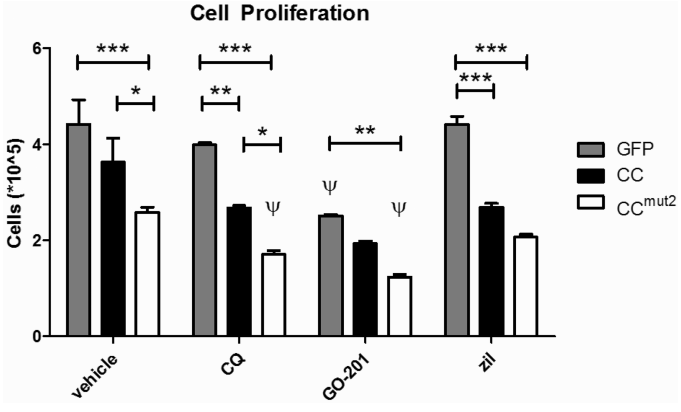

Figure 5. K562 cell proliferation as indicated by trypan blue dye exclusion reveals that transfected cells subsequently treated with drugs can further depress the proliferative capacity of K562 cells. CC^{mut2} has a significant effect alone, compared to that of the GFP control. Drug addition does not significantly affect GFP-treated cells, with the exception of GO-201. CC + drug and CC^{mut2} + drug have further reductions in proliferation compared to that of vehicle treated cells. GO-201 combined with CC^{mut2} has a potent reduction in proliferation compared to that of other drugs at 48 h. Two-way ANOVA and the Bonferroni post-test have the following significance levels: $*p < 0.05$, $**p < 0.01$; $***p < 0.001$, $n \geq 3$. ψ indicates at least $p < 0.05$ following a one-way ANOVA comparing the drug treated sample to the control (vehicle treated) in matched transfected groups, Bonferroni post-test.

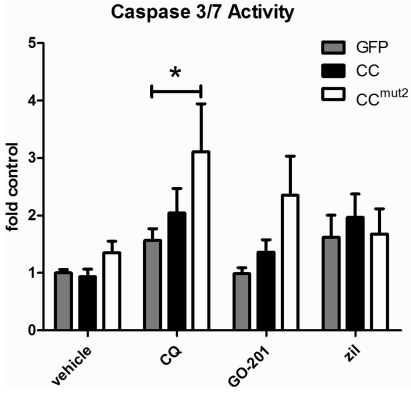

Figure 6. Apoptosis induction measured by activated caspase 3 or 7 is increased in CQ treated cells when comparing GFP control and CC_{mut2}. Data is expressed as fold vehicle control. Two-way ANOVA with Bonferroni post-tests. $n = 5$; $*p < 0.05$.

(Figure 7A) is reflected in the necrotic population at 72 h (Figure 7E). CC^{mut2} alone significantly increases apoptosis at 96 h (Figure 7C) and is significantly enhanced by the addition of CQ. While zil also increases apoptosis, it is not above vehicle control levels. Necrosis at 96 h appears only to be impacted by CC^{mut2} (Figure 7F).

DISCUSSION

In this study, we report that drug and transfection combinations can enhance elimination of K562 CML cells. Using a novel genetic therapy (CC^{mut2}), we demonstrated enhanced apoptotic cell death and reduced proliferation ability of CC^{mut2} expressing K562 cells consistent with previous reports.[4,12] We then evaluated the transformative and proliferative capacity of cells using a combination of constructs or drugs focused on inhibiting BCR-ABL signaling and other leukemia-specific pathways. Though we did not see a significant reduction in transformative ability with the transfection of a single plasmid alone, the combination of constructs targeting Alox5 knockdown with CC^{mut2} elucidated the importance of Alox5 in long-term cell survival and colony forming ability (Figure 3). Previous studies involving Alox5 in CML do not address the transformative ability of K562 cells but focus more on stem-like cells in *in vivo* models.[5,15,24,31,37] Though other assays did not indicate a robust role for Alox5 in apoptosis or proliferation, the fact that Alox5 could be an important target in blast-phase CML cells in addition to a primordial CML stem cell is an interesting observation.

We also demonstrated further reduction in proliferative capacity resulting from the combination of GO-201 or CQ and CC^{mut2} transfections (Figure 5). Additionally, effector caspase 3 and 7 activation increased following the combination with CQ at 48 h (Figure 6). Finally, combining CQ and CC^{mut2} appear to enhance apoptosis/necrosis when both exist in the cell long enough to have an effect individually (Figure 7). Interestingly, increases in apoptosis and necrosis were primarily due to the small molecule drug (CQ) or peptide therapeutic (GO-201) (Figure 7A, D), then later affected by the transfection (Figure 7B–C and E–F). This indicates a temporal shift in the activity and effect of the therapeutic, which may become important in future studies. Together, these data suggest further increases are achievable using a second agent in addition to the therapeutic interfering peptide CC^{mut2}. Specifically, combinations with CC^{mut2} and CQ appear to have a broad effect on proliferation and apoptosis, followed by GO-201. In fact, the hydroxychloroquine derivative of CQ is currently being investigated as an adjuvant to IM therapy for CML in a clinical study (NCT01227135). Future studies will evaluate the most potent combinations in primary patient samples, including stem-like CML cells. Importantly, this article demonstrates the efficacy of a combination based approach using small molecule or peptide-based inhibitors to target both the causative oncogenic BCR-ABL but also key alternative pathways in a blast-crisis CML cell line.

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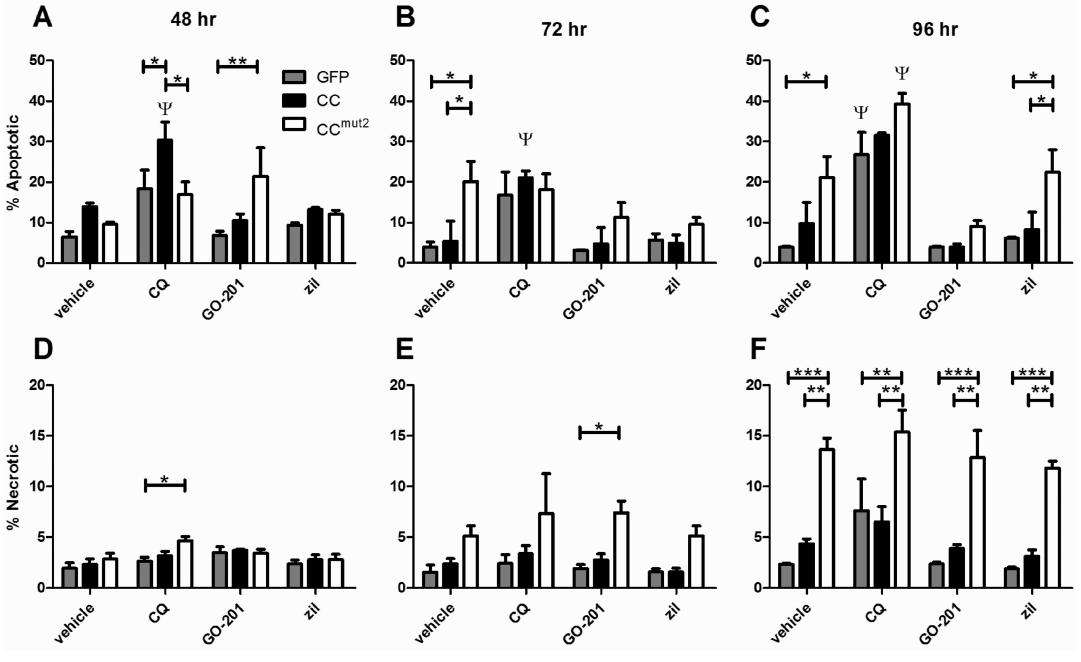

Figure 7. Transfected and treated cells were subjected to Annexin-V (apoptotic, A–C) and 7-AAD (necrotic, D–F) analysis at 48, 72, and 96 h. Drug treated populations have a larger initial effect on apoptosis and necrosis, followed by a transfection-caused increase in apoptosis beginning at 72 h. GO-201 significantly shifts the CC^{mut2} treated cells toward apoptosis at 48 h, and this affect can be seen to reflect an enhanced necrotic population at 72 h within the GO-201 group; however, neither of these is significantly different from the untreated control. A significant apoptotic increase is observed in the vehicle conditions including in the CC^{mut2} transfected cells after 72 h, translating to increased necrosis at 96 h in all conditions, due only to CC^{mut2}. The CQ treated group shows an early enhancement of apoptosis (48–72 h) compared to that of the vehicle control in the CC group. At 96 h, within the CQ treatment group, both GFP and CC^{mut2} are significantly increased compared with thos of the vehicle control. $n = 3$; two-way ANOVA with Bonferroni post-test, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. ψ indicates at least $p < 0.05$ following a one-way ANOVA comparing the drug treated sample to the control (vehicle treated) in matched transfected groups, Bonferroni post-test.

■ ASSOCIATED CONTENT

Supporting Information

Statistical details for 2-way ANOVA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CML, chronic myeloid leukemia; BCR, breakpoint cluster region; ABL, Abelson; TKI, tyrosine kinase inhibitor; ATP, adenosine-5′-triphosphate; LSC, leukemic stem cell; IM, imatinib; CC, coiled-coil; RNAi, RNA interference; shRNA, short-hairpin RNA; GFP, enhanced green fluorescent protein; LC3, light chain-3; zil, zileuton; CQ, chloroquine

■ REFERENCES

(1) Bartram, C. R.; de Klein, A.; Hagemeijer, A.; van Agthoven, T.; Geurts van Kessel, A.; Bootsma, D.; Grosveld, G.; Ferguson-Smith, M. A.; Davies, T.; Stone, M.; et al. Translocation of c-ab1 oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. *Nature* **1983**, *306* (5940), 277–280.

(2) O'Hare, T.; Eide, C. A.; Deininger, M. W. Bcr-Abl kinase domain mutations, drug resistance, and the road to a cure for chronic myeloid leukemia. *Blood* **2007**, *110* (7), 2242–2249.

(3) Woessner, D. W.; Lim, C. S.; Deininger, M. W. Development of an effective therapy for chronic myelogenous leukemia. *Cancer J.* **2011**, *17* (6), 477–486.

(4) Dixon, A. S.; Pendley, S. S.; Bruno, B. J.; Woessner, D. W.; Shimpi, A. A.; Cheatham, T. E., III; Lim, C. S. Disruption of Bcr-Abl coiled coil oligomerization by design. *J. Biol. Chem.* **2011**, *286* (31), 27751–27760.

(5) Chen, Y.; Li, D.; Li, S. The Alox5 gene is a novel therapeutic target in cancer stem cells of chronic myeloid leukemia. *Cell Cycle* **2009**, *8* (21), 3488–3492.

(6) Kawano, T.; Ito, M.; Raina, D.; Wu, Z.; Rosenblatt, J.; Avigan, D.; Stone, R.; Kufe, D. MUC1 oncoprotein regulates Bcr-Abl stability and pathogenesis in chronic myelogenous leukemia cells. *Cancer Res.* **2007**, *67* (24), 11576−11584.
(7) Bellodi, C.; Lidonnici, M. R.; Hamilton, A.; Helgason, G. V.; Soliera, A. R.; Ronchetti, M.; Galavotti, S.; Young, K. W.; Selmi, T.; Yacobi, R.; Van Etten, R. A.; Donato, N.; Hunter, A.; Dinsdale, D.; Tirro, E.; Vigneri, P.; Nicotera, P.; Dyer, M. J.; Holyoake, T.; Salomoni, P.; Calabretta, B. Targeting autophagy potentiates tyrosine kinase inhibitor-induced cell death in Philadelphia chromosome-positive cells, including primary CML stem cells. *J. Clin. Invest.* **2009**, *119* (5), 1109−1123.
(8) Rowley, J. D. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature* **1973**, *243* (5405), 290−293.
(9) Anafi, M.; Gazit, A.; Gilon, C.; Ben-Neriah, Y.; Levitzki, A. Selective interactions of transforming and normal abl proteins with ATP, tyrosine-copolymer substrates, and tyrphostins. *J. Biol. Chem.* **1992**, *267* (7), 4518−4523.
(10) Nicholson, E.; Holyoake, T. The chronic myeloid leukemia stem cell. *Clin. Lymphoma Myeloma* **2009**, *9* (Suppl. 4), S376−S381.
(11) Zhang, X.; Subrahmanyam, R.; Wong, R.; Gross, A. W.; Ren, R. The NH(2)-terminal coiled-coil domain and tyrosine 177 play important roles in induction of a myeloproliferative disease in mice by Bcr-Abl. *Mol. Cell. Biol.* **2001**, *21* (3), 840−853.
(12) Dixon, A. S.; Miller, G. D.; Bruno, B. J.; Constance, J. E.; Woessner, D. W.; Fidler, T. P.; Robertson, J. C.; Cheatham, T. E., III; Lim, C. S. Improved coiled-coil design enhances interaction with Bcr-Abl and induces apoptosis. *Mol. Pharmaceutics* **2012**, *9* (1), 187−195.
(13) Corbin, A. S.; Agarwal, A.; Loriaux, M.; Cortes, J.; Deininger, M. W.; Druker, B. J. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *J. Clin. Invest.* **2011**, *121* (1), 396−409.
(14) O'Hare, T.; Deininger, M. W.; Eide, C. A.; Clackson, T.; Druker, B. J. Targeting the BCR-ABL signaling pathway in therapy-resistant Philadelphia chromosome-positive leukemia. *Clin. Cancer Res.* **2011**, *17* (2), 212−221.
(15) Chen, Y.; Hu, Y.; Zhang, H.; Peng, C.; Li, S. Loss of the Alox5 gene impairs leukemia stem cells and prevents chronic myeloid leukemia. *Nat. Genet.* **2009**, *41* (7), 783−792.
(16) Yin, L.; Ahmad, R.; Kosugi, M.; Kawano, T.; Avigan, D.; Stone, R.; Kharbanda, S.; Kufe, D. Terminal differentiation of chronic myelogenous leukemia cells is induced by targeting of the MUC1-C oncoprotein. *Cancer Biol. Ther.* **2010**, *10* (5), 483−491.
(17) Warsch, W.; Kollmann, K.; Eckelhart, E.; Fajmann, S.; Cerny-Reiterer, S.; Holbl, A.; Gleixner, K. V.; Dworzak, M.; Mayerhofer, M.; Hoermann, G.; Herrmann, H.; Sillaber, C.; Egger, G.; Valent, P.; Moriggl, R.; Sexl, V. High STAT5 levels mediate imatinib resistance and indicate disease progression in chronic myeloid leukemia. *Blood* **2011**, *117* (12), 3409−3420.
(18) Naka, K.; Hoshii, T.; Hirao, A. Novel therapeutic approach to eradicate tyrosine kinase inhibitor resistant chronic myeloid leukemia stem cells. *Cancer Sci.* **2010**, *101* (7), 1577−1581.
(19) Imai, K.; Takaoka, A. Comparing antibody and small-molecule therapies for cancer. *Nat. Rev. Cancer* **2006**, *6* (9), 714−727.
(20) Kaelin, W. G., Jr. The concept of synthetic lethality in the context of anticancer therapy. *Nat. Rev. Cancer* **2005**, *5* (9), 689−698.
(21) Brossart, P.; Schneider, A.; Dill, P.; Schammann, T.; Grunebach, F.; Wirths, S.; Kanz, L.; Buhring, H. J.; Brugger, W. The epithelial tumor antigen MUC1 is expressed in hematological malignancies and is recognized by MUC1-specific cytotoxic T-lymphocytes. *Cancer Res.* **2001**, *61* (18), 6846−6850.
(22) Edinger, A. L.; Thompson, C. B. Death by design: apoptosis, necrosis and autophagy. *Curr. Opin. Cell Biol.* **2004**, *16* (6), 663−669.
(23) Livesey, K. M.; Tang, D.; Zeh, H. J.; Lotze, M. T. Autophagy inhibition in combination cancer treatment. *Curr. Opin. Invest. Drugs* **2009**, *10* (12), 1269−1279.
(24) Peng, C.; Chen, Y.; Shan, Y.; Zhang, H.; Guo, Z.; Li, D.; Li, S. LSK derived LSK(-) cells have a high apoptotic rate related to survival regulation of hematopoietic and leukemic stem cells. *PloS one* **2012**, *7* (6), e38614.
(25) Al Baghdadi, T.; Abonour, R.; Boswell, H. S. Novel combination treatments targeting chronic myeloid leukemia stem cells. *Clin. Lymphoma Myeloma Leuk.* **2012**, *12* (2), 94−105.
(26) Zhong, X. Y.; Kaul, S.; Bastert, G. Evaluation of MUC1 and EGP40 in bone marrow and peripheral blood as a marker for occult breast cancer. *Arch. Gynecol. Obstet.* **2001**, *264* (4), 177−181.
(27) Yin, L.; Ahmad, R.; Kosugi, M.; Kawano, T.; Avigan, D.; Stone, R.; Kharbanda, S.; Kufe, D. Terminal differentiation of chronic myelogenous leukemia cells is induced by targeting of the MUC1-C oncoprotein. *Cancer Biol. Ther.* **2010**, *10* (5), 483−491.
(28) Radmark, O.; Werz, O.; Steinhilber, D.; Samuelsson, B. 5-Lipoxygenase: regulation of expression and enzyme activity. *Trends Biochem. Sci.* **2007**, *32* (7), 332−341.
(29) Snyder, D. S.; Castro, R.; Desforges, J. F. Antiproliferative effects of lipoxygenase inhibitors on malignant human hematopoietic cell lines. *Exp. Hematol.* **1989**, *17* (1), 6−9.
(30) Tsukada, T.; Nakashima, K.; Shirakawa, S. Arachidonate 5-lipoxygenase inhibitors show potent antiproliferative effects on human leukemia cell lines. *Biochem. Biophys. Res. Commun.* **1986**, *140* (3), 832−836.
(31) Chen, Y.; Peng, C.; Sullivan, C.; Li, D.; Li, S. Novel therapeutic agents against cancer stem cells of chronic myeloid leukemia. *Anticancer Agents Med. Chem.* **2010**, *10* (2), 111−115.
(32) Ertmer, A.; Huber, V.; Gilch, S.; Yoshimori, T.; Erfle, V.; Duyster, J.; Elsasser, H. P.; Schatzl, H. M. The anticancer drug imatinib induces cellular autophagy. *Leukemia* **2007**, *21* (5), 936−942.
(33) Han, W.; Pan, H.; Chen, Y.; Sun, J.; Wang, Y.; Li, J.; Ge, W.; Feng, L.; Lin, X.; Wang, X.; Jin, H. EGFR tyrosine kinase inhibitors activate autophagy as a cytoprotective response in human lung cancer cells. *PloS One* **2011**, *6* (6), e18691.
(34) Mizushima, N.; Yoshimori, T.; Levine, B. Methods in mammalian autophagy research. *Cell* **2010**, *140* (3), 313−326.
(35) Mizushima, N.; Yoshimori, T. How to interpret LC3 immunoblotting. *Autophagy* **2007**, *3* (6), 542−545.
(36) Bayascas, J. R.; Yuste, V. J.; Benito, E.; Garcia-Fernandez, J.; Comella, J. X. Isolation of AmphiCASP-3/7, an ancestral caspase from amphioxus (*Branchiostoma floridae*). Evolutionary considerations for vertebrate caspases. *Cell Death Differ.* **2002**, *9* (10), 1078−10789.
(37) Sullivan, C.; Peng, C.; Chen, Y.; Li, D.; Li, S. Targeted therapy of chronic myeloid leukemia. *Biochem. Pharmacol.* **2010**, *80* (5), 584−591.

Supporting Information

Statistical Details for two-way ANOVA. Figure 3.3: two-way ANOVA p-values: no significant overall interaction between short-hairpin transfection and coiled-coil transfection (p=0.3480). The transfection (GFP, CC, or CC^{mut2}) significantly affects colony formation (p=0.0010). Knockdown transfection (shLuc, shAtg7, shMUC-1, shAlox5) also affects colony forming cells (p=0.0162).

Figure 3.5: two-way ANOVA p-values: no significant overall interaction (p=0.2042), with both transfection and drug treatment significantly affecting the result (p<0.0001).

Figure 3.6: two-way ANOVA p-values: no significant overall interaction between drug treatment and transfection (p=0.3749). Transfection significantly affected caspase activity (p=0.0129) as did drug treatment (p=0.0087).

Figure 3.7: two-way ANOVA p-values: A) there is a significant overall interaction (p=0.0252), making the following p-values difficult to interpret. Transfection does significantly affect the result (p=0.0130). Drug treatment group significantly affects the result (p=0.0003). B) No significant overall interaction (p=0.2462). Transfection significantly affects the result (p=0.0146). Drug treatment group significantly affects the result (p=0.0006). C) No significant overall interaction (p=0.7156). Transfection significantly affects the result (p=0.0003). Drug treatment group significantly affects the result (p<0.0001). D) No significant overall interaction (p=0.3873). Transfection does not significantly affect the result (p=0.0590). Drug treatment group significantly affects the result (p=0.0098). E) No significant overall interaction (p=0.9854). Transfection significantly affects the result (p=0.0002). Drug treatment group does not significantly

affects the result ($p=0.4144$). F) No significant overall interaction ($p=0.9397$). Transfection significantly affects the result ($p<0.0001$). Drug treatment group significantly affects the result ($p=0.0176$). This information is available free of charge via the Internet at http://pubs.acs.org/

CHAPTER 4

DISRUPTION OF BCR-ABL1 DIMERIZATION FOR CHRONIC MYELOID LEUKEMIA THERAPY: RETARGETING THERAPEUTICS IN NORMAL AND TKI-RESISTANT CELLS[1]

Abstract

Targeted therapy for chronic myeloid leukemia is centered on direct inhibition of the tyrosine kinase domain by small molecule therapeutics. This strategy has been successful in treating the majority of disease, but is subject to drug evasion following kinase domain mutations. Kinase activity results from transactivation of BCR-ABL1 following an oligomerization event. The responsible and necessary domain for oligomerization is the CC domain in the N-terminus of BCR. Here we describe how a preferential and specific engineered mutant version of CC, the CCmut3, can be introduced as a therapeutic to limit oncogenic properties of the BCR-ABL1 containing cell. Analysis of apoptosis induction, proliferation, and transformative ability all demonstrate strong therapeutic potential in cell lines containing either wild-type p210 BCR-ABL1, single kinase domain mutations (E255V and T315I), and in a compound (E255V/T315I) BCR-ABL1 kinase mutant cell line. Finally, we show efficacy of colony forming inhibition in patient samples from individuals with newly diagnosed chronic

[1] Submitted to *Leukemia*

myeloid leukemia and one harboring the T315I mutation.

Introduction

The BCR-ABL1 reciprocal translocation t(9;22) (q34;q11) is the transforming event in converting hematopoietic cells into leukemia cells and the known cause of chronic myeloid leukemia (CML) (1, 2). Constitutive activity from the ABL1 tyrosine kinase in the fusion oncoprotein is a hallmark of CML and the specific target of small molecule therapeutics for the disease including the first inhibitor of its kind, imatinib (3-5). Overall, tyrosine kinase inhibitors (TKIs) have displayed outstanding efficacy in CML, reducing disease burden, measured by complete hematologic (CHR) and cytogenetic response (CCyR), directly resulting in increased progression-free and overall survival rates compared to those before the introduction of TKIs (6-11). However, acquired kinase domain mutations confer BCR-ABL1-dependent resistance and have presented a significant barrier to TKI efficacy (12), leading to development of the next-generation TKIs dasatinib, nilotininb and bosutinib and most recently the pan-BCR-ABL inhibitor ponatinib (13).

Ponatinib is notably the only effective agent for patients with the T315I mutation, a major breakthrough in disease treatment (14). Second generation TKIs produced a more reliable response to imatinib-resistant patients who carried any BCR-ABL1 mutation, producing CCyR rates of 47% and 32% for dasatinib and nilotinib, respectively (15). However, nilotinb failed to produce a CCyR in a majority of P-loop mutants including E255V. Additionally, although dasatinib treatment was able to produce a CCyR of 36% in patients with the E255V mutation, neither dasatinib nor nilotinib effectively inhibit the

T315I BCR-ABL1 gatekeeper mutation (15, 16). Interestingly, preliminary reports indicate patients can still acquire kinase mutations, including the E255V mutant, during ponatinib therapy, the long-term outcome of which is still unknown (17). Therapeutic efficacy becomes less clear with the development of multiple mutations within a single BCR-ABL1 gene, known as compound mutants in CML. The lineage of these are just now beginning to be understood (18).

While due focus has been given to ABL1-directed kinase inhibitors for CML, design of these small molecule inhibitors inevitably leads to increased propensity for selection of kinase domain mutations which inactivate drug binding (19). Few alternatives to inhibiting BCR-ABL1 activity and maintaining selectivity for the molecule have been proposed. One early proposal made by Ruthardt et al. described how inhibition of BCR-ABL1 oligomerization could block transactivation of ABL1 (20). By isolating and artificially introducing a mimic of the critical dimerization motif, helix α2 residing on the N-terminal coiled-coil (CC) domain in BCR, Ruthardt et al. demonstrated reduced phosphorylation of BCR-ABL1 and efficacy in other assays (21). However, the isolated wild-type helix 2 was inactive in the T315I mutant cell line (21, 22).

Recently we described two iterations of a mutant CC (called CC^{mut2} or CC^{mut3}) with preferential specificity toward heterooligomerization with the CC of BCR (over homooligomerization with itself) (23, 24). This construct is similar to the Ruthardt helix 2, but contains the full-length CC domain with engineered mutations for binding specificity within helix 2. Our previous results demonstrate significant inhibitory activity against wild-type p210 BCR-ABL1 containing K562 cells (23, 24), but BCR-ABL1 mutant cell lines and patient-derived samples have not yet been examined. We

hypothesized that: 1) the efficacy of CC^{mut3} will not be affected by kinase domain mutation in BCR-ABL1, which inactivates current TKIs in vitro; and that 2) patient samples, regardless of kinase domain mutations, would also be susceptible to inhibition of BCR-ABL1 by CC^{mut3}. To evaluate these hypotheses we studied the effect of CC^{mut3} on Ba/F3 cells transduced with p210-BCR-ABL1 on induction of apoptosis, proliferation, and colony formation. We also performed the first analysis of CC^{mut3} efficacy in primary samples from newly diagnosed and TKI-resistant CML patients.

Materials and Methods

DNA Constructs

pmCherry-EV (empty vector) and pmCherry-CC^{mut3} have been described previously (24).The lentivirus control vector pCDH-EF-copGFP-EV was adapted from pCDH-CMV-MCS-EF1-copGFP (System Biosciences (SBI), Mountain View, CA). The CMV promoter and MCS were excised using the restrictions endonucleases SpeI and XbaI with compatible cohesive ends. The CMV fragment was removed using gel purification and the resulting DNA was ligated to form the final construct.

To make pCDH-EF-copGFP-CC^{mut3}, sections of the construct were amplified separately by PCR and knit together using overlap extension PCR. First, EF1-copGFP was amplified from the SBI parent plasmid with a 5'SpeI and 3'BamHI site using the following primers: 5'-CAACTAGTAAGGATCTGCGATCGCTCC-3' and 5'-CCAT CTGAGTCCGGAGCGAGATCCGGTGGAGC-3.' CC^{mut3} was amplified from pEGFP-CC^{mut3} described in (24) using the following primers containing a 5'BamHI site, a terminal TAG stop signal and a sequence complimentary to the polyA signal on the 3'

overhang: 5'-CTCAGATGGATCCTTATGGTGGACCCGGTGGGCTTCG-3' and 5'-GTTATCTAGATCTACCGGTCATAGCTCTTCTTTTCC-3'. Finally, the polyA signal from pEGFP-C1 (Clontech Laboratories, Mountain Veiw, CA) was amplified to include a 5' complimentary sequence to CC^{mut3}, and a 3' SalI restriction site using these primers 5'-GACCCGGTAGATCTAGATAACTGATCATAATC-3' and 5'-GCTTACATGCGG CCGCGTCGACTGTGGGAGGTTTTTTAAAGC-3.' PCR products were combined in two steps, first by combining the CC^{mut3}-polyA and then by adding EF-copGFP by overlap extension PCR. The PCR product was digested with SpeI and SalI and ligated to the pCDH-CMV-MCS-EF1-copGFP vector (SBI) also cut with SpeI and SalI.

psPAX2 was purchased from Cellecta, Inc. (Mountain View, CA) and pVSV-G was purchased from Clontech (Mountainview, CA).

Cell Lines, Transfections, and Lentivirus Generation

Stable recombinant Ba/F3 cells transduced with wild-type p210 BCR-ABL1 (25), the kinase domain mutants p210-T315I, p210-E255V, or the compound mutant p210-E255V-T315I were generated in the Deininger laboratory (26, 27). These cells were cultured in RPMI with 10% FBS, 1% penicillin, streptomycin, glutamine, and 0.1% gentamycin (RPMI complete). Additionally 0.1% MycoZap™ (Lonza Bio, Basel, Switzerland) was added to prevent possible mycoplasma contamination. The non-transduced parental Ba/F3 cell line was grown in RPMI complete with 20% IL-3 conditioned media generated using WEHI-3B cells (28), also grown in RPMI complete. Ba/F3 cells were transfected with plasmid DNA using the Amaxa nucleofection system (Lonza Bio). Four micrograms of plasmid DNA was added to 3 million cells in 100 μL of

solution V with supplement and nucleofected using program X-001. Cells were returned to plain RPMI for 20 minutes and then transferred to 3 mL of RPMI complete for 24 h. Cells were sorted on a BD FACSAria cytometer (BD Biosciences, San Jose, CA) for double-positive cells expressing mCherry and GFP then returned to RPMI complete for subsequent experiments.

293-FT cells (Life Technologies, Grand Island, NY) were grown in DMEM with 10% FBS (Atlanta Biologicals, Atlanta, GA), 1% penicillin, streptomycin, glutamine, MEM-nonessential amino acids, and sodium pyruvate (Life Technologies). Cells were passaged every 2-3 days in T75 flasks, and grown to 65% confluence in T-175 flasks for transfection.

For lentivirus generation, cells were transfected with 30 μg of the experimental construct pCDH-EF-copGFP-EV or pCDH-EF-copGFP-CC^{mut3}, 5 μg of VSVG and 8 μg of psPAX2 using the Profection® mammalian transfection reagent (Promega, Madison, WI) according to manufacturer instructions. The DNA solution in 3 mL was then added drop-wise to T175 flasks containing 293-FT cells. After 48 h of transfection, viral particles were complexed with PEG overnight, pelleted, and resuspended in RPMI complete. Lentiviral titer was performed by seeding 50,000 293-FT cells per well in a 24-well plate and adding serial dilutions of virus to the cells. Cells with GFP fluorescence were detected on a Guava easyCyte HT cytometer (EMD Millipore, Billerica, MA), and positive cells to total cell number was used for titer calculation.

Patient Samples and Lentivirus Infection

Mononuclear cells (MNCs) from peripheral blood (PB) of patients with newly diagnosed CML or those with a documented T315I mutation were used in these studies. Prior to use in assays, cells were Ficoll-separated and used for automated isolation of the $CD34^+$ fraction using an autoMACS Pro (Miltenyi Biotech). Fresh or frozen $CD34^+$ progenitors from CML patients were kept at 1E6 cells/mL when possible in RPMI-complete containing 20% FBS and 5 μL/mL StemSpan CC100 (Stem Cell Technologies, Vancouver, BC, Canada). Cells were infected with lentivirus at a MOI of 5 for each construct at 24 and 48 h following harvest (fresh) or thaw (frozen). Cells were subject to fluorescence-activated cell sorting on a BD FACSAria cytometer after 72 h, and GFP positive cells were returned to culture in RPMI complete with 5 μL/mL CC100 for future experiments. All patients gave their informed consent in accordance with the Declaration of Helsinki, and all studies with human specimens were approved by The University of Utah Institutional Review Board (IRB).

Proliferation

Cell proliferation was assessed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) assay (Promega) according to manufacturer instructions. Briefly, 5,000 viable cells were added to a single well in 96-well plate in 100 μL RPMI complete medium. Three independent samples were seeded in duplicate for each time point. Cells were allowed to grow for 3 or 4 days and read at 490 nM on a SpectraMax M2 plate reader (Molecular Devices,Sunnyvale, CA) 3 h after incubation with the MTS reagent.

Apoptosis and Cell Death

Apoptosis and cell death were assessed via flow cytometry using a BD FACSCanto analyzer as described previously (29). Cell lines were pelleted and resuspended in AnnexinV-binding buffer (BD Biosciences) then analyzed following addition of the apopototic marker AnnexinV-APC (BD Biosciences or Life Technologies) and cell impermeable nuclear dye 7-AAD (BD Biosciences or Life Technologies). In addition to the APC or 7-AAD channel, events that were GFP and mCherry positive were also recorded. A minimum of 500 events were recorded.

Methylcellulose Colony Formation

Following selection of transfected cells by cell sorting, cells were counted using trypan blue exclusion and seeded into a methylcellulose colony forming assay (CFA) as described previously (23). Briefly, mCherry-positive Ba/F3 cell lines were resuspended in IMDM with 2% FBS (Stem Cell Technology) at a concentration of 10,000 cells/mL. Three hundred microliters of this dilution was added to 3 mL of Methocult media (M3434 – Ba/F3 p210 wild-type and mutant lines or M3234 – Ba/F3 parental, Stem Cell Technologies). Approximately 1,100 cells, or 1.2 mL, were seeded per dish in duplicate for each transfection. Colonies were counted 7 days later in an area of 100 μm^2 per dish.

Primary cells were seeded in Methocult H4230 (Stem Cell Technologies) as described previously (30). Briefly, lentivirus-infected (GFP+) sorted cells were counted by trypan blue exclusion and resuspended at a concentration of 5,000 cells/mL into RPMI complete. Four hundred forty microliters of this dilution was added to 1.8 mL of methocult with 11 μL of CC100 cytokines and with or without TKI as indicated in each

experiment. Approximately 1,000 cells, or 1.1 mL, were seeded per plate. Plates were incubated at 37C in 5% CO_2 in a humidified incubator for 14 days. Hematopoietic colonies were scored by standard morphologic criteria using an inverted microscope.

Cell Growth

Lentivirus infected and sorted primary cells were counted by trypan blue exclusion and seeded at 10,000 cells per well in a 96-well plate following cell sorting. Cells were counted beginning on day 2, daily through day 7 to examine cell growth of each individual sample when sufficient number was available. TKIs imatinib (at 0 or 2.5 μM for ND CML samples) or ponatinib (at 0, 10, 25, or 50 μM for T315I CML samples) were added at days 0 and 4 as controls when sufficient cell number was available.

Results

CC^{mut3} Universally Potentiates Apoptosis of Ba/F3 Cells with p210 BCR-ABL1

Apoptosis was measured by flow cytometry at 72 h following transfection of either empty vector (EV) or CC^{mut3} constructs tagged with the mCherry fluorescent protein. Cell events which were AnnexinV positive and 7AAD negative are considered the purely apoptotic population (31, 32). This population is reported in Figure 4.1 for each cell line. Transfection of EV and CC^{mut3} did not differentially induce apoptosis in the parental Ba/F3 cell line lacking BCR-ABL1 expression (Figure 4.1a). Ba/F3 cells stably expressing wild-type p210 BCR-ABL1 show a significant induction of approximately 3-fold when transfected with CC^{mut3} compared to EV (Figure 4.1b). The

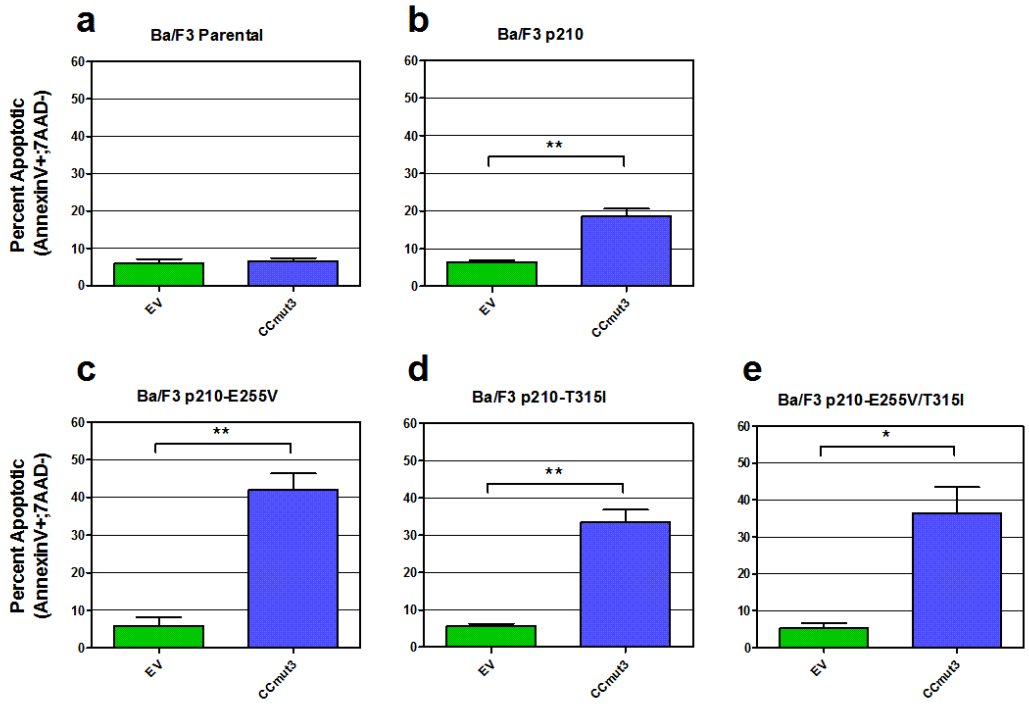

Figure 4.1. **CC^{mut3} universally enhances apoptosis of Ba/F3 cells with p210 BCR-ABL1.** Flow cytometric analysis of transfected and sorted cells for apoptotic cell population at 72 h are displayed. Apoptotic and necrotic stains AnnexinV and 7AAD reveal (a) Ba/F3 parental cells are not affected by overexpression of the CC^{mut3} compared to EV control. (b) Ba/F3 p210 cells have a more than 30% increase in apoptotic population when treated with CC^{mut3} vs EV control. Single Ba/F3 p210 mutants (c) E255V and (d) T315I display a significant shift toward the apoptotic fraction when expressing CC^{mut3} compared to EV. (e) Finally, the Ba/F3 p210 compound mutant E255V/T315I line is also significantly more apoptotic when treated with CC^{mut3}. n=3. Graphs display mean with error bars indicating S.E.M. Unpaired t-test significance levels are *$p<0.05$, **$p<0.01$.

CC^{mut3} transfection is also effective in increasing apoptosis in single mutant BCR-ABL1 cell lines (Figure 4.1c and 1d) similar to that of those with wild-type BCR-ABL (compare Figure 4.1b to c and d). When both ABL1 kinase domain mutations (E255V and T315I) are on the same molecule as a compound mutant (Ba/F3 p210-E255V/T315I), the CC^{mut3} still demonstrates ability to induce apoptosis (Figure 4.1e).

Proliferation of BCR-ABL1-Expressing Cells Is Inhibited by CC^{mut3} Expression

The MTS tetrazolium compound, which is bioreduced by cellular metabolic processes, was used to determine the proliferative capacity of Ba/F3 cells with or without BCR-ABL1 following EV or CC^{mut3} transfection (33). In BCR-ABL1-null cells, there was no difference in proliferation at either 72 or 96 h following transfection (Figure 4.2a). However, Ba/F3 cells with p210 BCR-ABL did show sensitivity to expression of CC^{mut3} at 96 but not 72 h (Figure 4.2b). Single p210 BCR-ABL1 mutants demonstrated a small, yet significant inhibition of proliferation in the CC^{mut3} group compared with EV at 72 h which continued to limit proliferation to a greater extent at 96 h when compared with EV expression in the same cell lines (Figure 4.2c and 2d). Ba/F3 compound mutant cells show a much diminished difference between EV and CC^{mut3} at 96 h when compared with other lines, including those with single mutations. A detectable difference is seen at 96 but not 72 h.

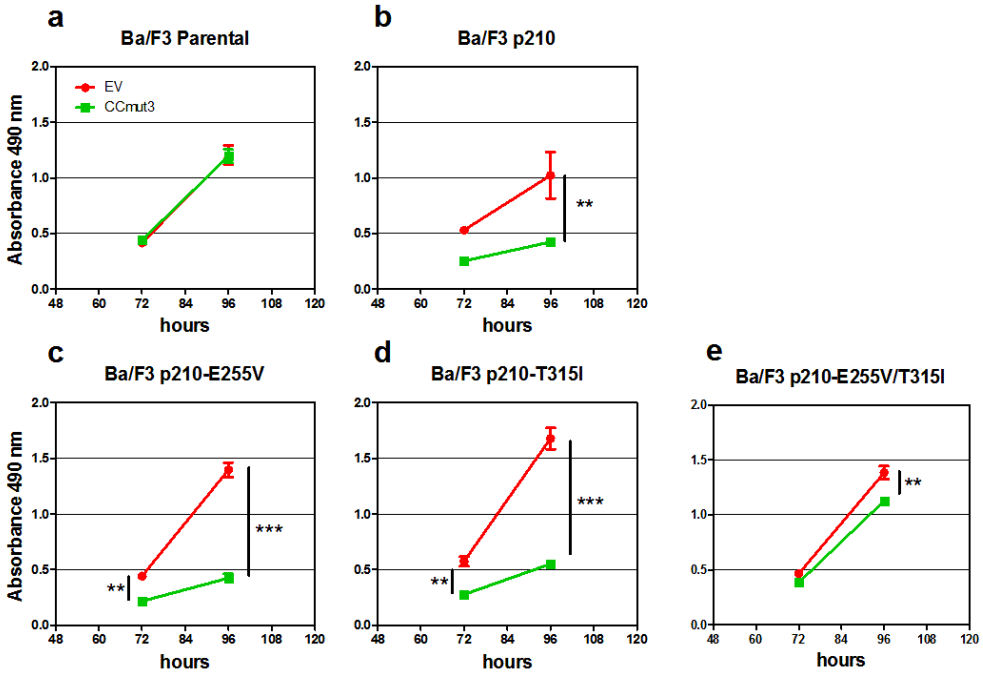

Figure 4.2. **Proliferation of BCR-ABL1 expressing cells is inhibited by CC^{mut3} expression**. (a) Ba/F3 parental cells were not affected by overexpression of the CC^{mut3} compared to EV control. (b) CC^{mut3} treatment of Ba/F3 p210 cells significantly reduced proliferation at 96 but not 72 h compared to EV control. (c) CC^{mut3} but not EV expression results in significant reduction of single Ba/F3 p210 mutants E255V and (d) T315I proliferative capacity at both 72 and 96 h time points. (e)The compound mutant line containing both E255V and T315I shows a smaller but significant shift at 96 h when comparing CC^{mut3} to EV, but no difference in proliferation at 72 h. n=3. Graphs display mean with error bars indicating S.E.M. two-way ANOVA was used to compare differences with p-values generated by a Tukey's posttest. **p<0.01, ***p<0.001.

Colony Forming Cells (CFCs) Are Significantly Decreased Following Expression of CC^{mut3}

A methylcellulose colony forming assay was used to define the transformation potential of cells transfected with either EV or CC^{mut3}. As in previous assays, the Ba/F3 parental cell line displayed no difference between expression of EV or CC^{mut3} (Figure 4.3a). However, when the wild-type p210 BCR-ABL1 protein is present, CC^{mut3} almost completely eliminated CFCs when compared to the EV control (Figure 4.3b). Similarly, Ba/F3 E255V (Figure 4.3c) and Ba/F3 T315I (Figure 4.3d) also produced a greater than 10-fold reduction in CFCs in the CC^{mut3} group compared to control treatment. Finally, expression of CC^{mut3} in the compound mutant cell line was able to reduce CFCs by about half compared to EV control (Figure 4.3e).

Cells From Newly Diagnosed CML Patients Are Sensitive to CC^{mut3} Lentiviral Therapy Ex Vivo

Mononuclear cells isolated from peripheral blood of newly diagnosed CML patients and enriched for CD34 (Table 4.1) were transduced with EV or CC^{mut3}-expressing lentivirus. Following sorting for infected cells, patient samples were seeded into a cell growth assay or colony forming assay. Viable cells determined by trypan blue exclusion over a 7-day period were split into three groups: EV, EV+IM and CC^{mut3}. Viable cell numeration shows steady growth of the control (Figure 4.4a, circles). Addition of 2.5 μM IM to the EV-transduced cells on days 0 and 4 diminished growth uniformly on days 5-7 (Figure 4.4a, boxes). A comparable reduction in growth was also seen in the CC^{mut3} infected cells on days 5-7 (Figure 4.4a, triangles) and notably this

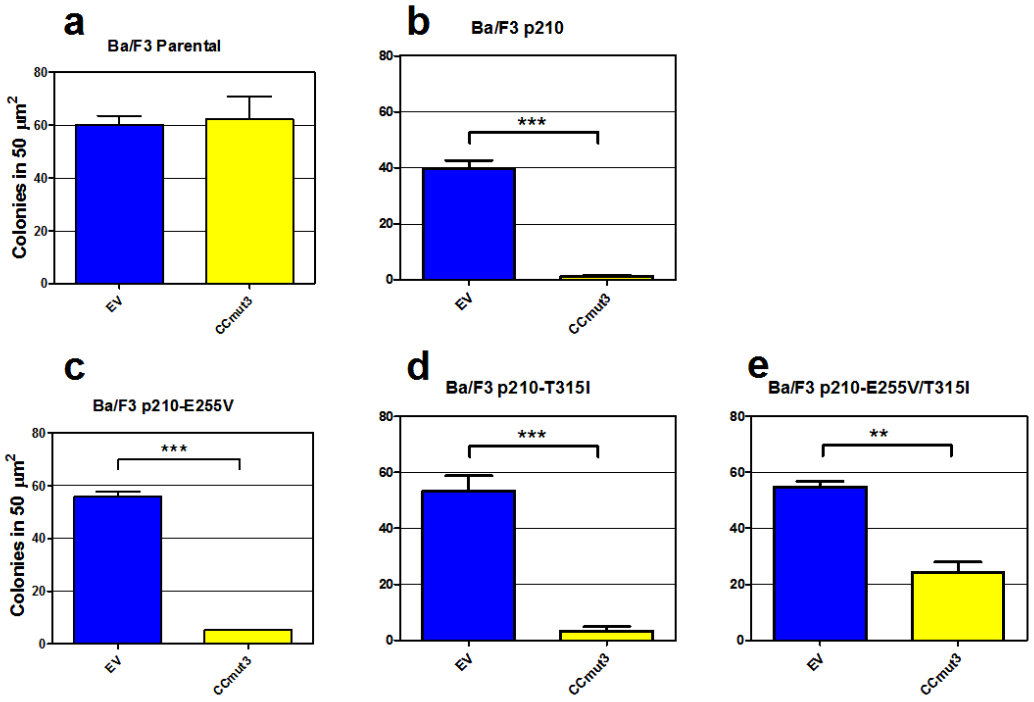

Figure 4.3. **Colony forming cells are significantly decreased following expression of CC^{mut3}**. (a) Ba/F3 parental cells are not affected by overexpression of the CC^{mut3} compared to EV control. (b) Transformative ability of Ba/F3 cells expressing p210 BCR-ABL1 is greatly reduced CC^{mut3} treatment group. Likewise in the Ba/F3 p210 mutants (c) E255V and (d) T315I, more than 10-fold fewer colonies are present in the CC^{mut3} group. (e) Colonies per area are again reduced in the compound mutant cell line (Ba/F3 p210-E255V/T315I) by CC^{mut3} compared to empty vector control, here by about 3-fold. n=3. Graphs display mean with error bars indicating S.E.M.; unpaired t-test significance levels are **p<0.01, ***p<0.001

Table 4.1. Patient Metrics for Ex Vivo Experiments With Lentiviral Transduction

Sample ID	Name	Age	Gender	Disease Status	Disease Phase	Mutation Status	Current/Previous Therapy
11-310	ND1	8	F	ND CML	Chronic	-	-
13-105	ND2	29	M	ND CML	Chronic/Accel .	-	-
12-072	ND3	44	F	ND CML	-	-	-
12-291	ND4	72	M	ND CML	Chronic	-	-
12-187	R1	66	F	IM, DAS Resistant	Accel.	T315I	Hydrea, anagrelide, INF/IM,DAS
13-004	R2	67	F	PON Resistant	Blast Crisis	T315I	PON/IM, DAS

Peripheral blood from CML patients with newly diagnosed (ND) CML or T315I resistant CML was collected and enriched for >90% CD34+ cells. The metrics presented here provide insight into the disease stage and treatment history of the patient samples used in this study. R1 and R2 are from the same patient at two different time points. Abbreviations: INF, peg-interferon, PON, ponatinib; Accel., accelerated; IM, imatinib; DAS, dasatinib.

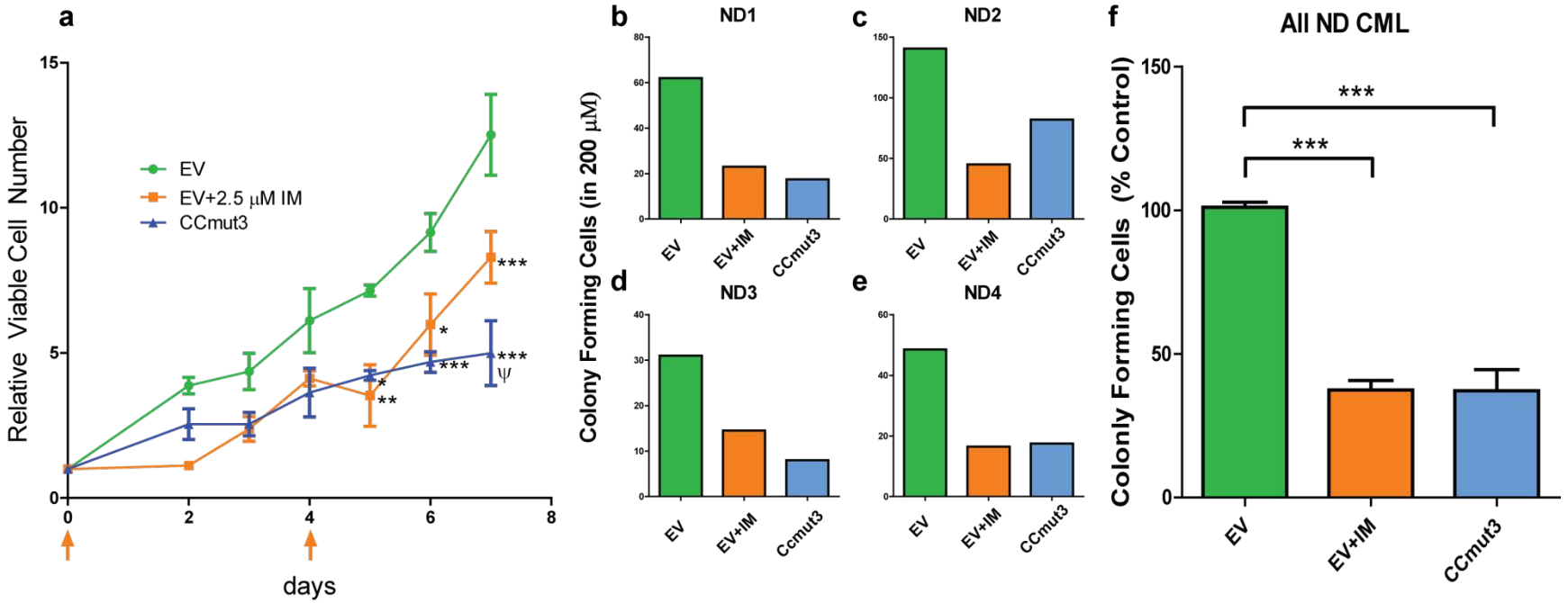

Figure 4.4. **Newly diagnosed CML patients are sensitive to CC^{mut3} lentiviral therapy ex vivo**. (a) Equal number of cells seeded was normalized to starting seed number on day 1 and plotted as fold-control represented by viable cells. Cell growth over 7 days is not significantly different for the 4 four days; beginning on the 5th day both IM treated and CC^{mut3} transduced cells display much slower growth through day 7. Additionally, CC^{mut3} transduced cells show an additional delayed growth compared to IM on the 7th day. n=3, graphs display mean with error bars indicating S.E.M. two-way ANOVA was used to compare differences with p-values generated by a Tukey's posttest, *p<0.5, **p<0.01, ***p<0.001, $^{\Psi}$p<0.5 compared to EV+IM group. (b-e) Colony forming cells were assessed 14 days following seeding in methylcellulose. ND1-4 are all sensitive to 2.5 μM IM and also when transduced with CC^{mut3} compared with EV control. Graphs represent individual samples counted in duplicate for each treatment. (f) Data from b-e were normalized to the EV control and compared. 2.5 μM IM and CC^{mut3} appear to be equally effective in reducing colony number. n=4, Graphs display mean with error bars indicating S.E.M. One-way ANOVA was used to compare differences with p-values generated by Tukey's post-test, ***p<0.001.

decrease appears to be persistent and more durable than transient IM addition. On day 7, CC^{mut3} treated cells were not only different from EV control, but also the IM treated group (Figure 4.4a, day 7 ψ).

When infected cells were introduced into methylcellulose with growth factors, individual patient samples appeared to respond to both IM and CC^{mut3} similarly (Figure 4.4b-e) with the exception of ND2 (Figure 4.4c), which had slightly more CFCs compared to EV+IM. All showed a marked reduction when compared with EV control. When data from ND1-4 were pooled and means compared, a significant (>50%) reduction from EV control was observed in both EV+IM and CC^{mut3} (Figure 4.4f).

CC^{mut3} Can Effectively Inhibit T315I Mutant BCR-ABL1-Driven Transformation In Accelerated But Not Blast Crisis CML *Ex Vivo*

Samples R1 and R2 represent cells from the same individual at two time points approximately 6 months apart (Table 4.1). Following infection with EV or CC^{mut3}, cells were seeded in a methylcellulose colony assay. For comparison, ponatinib at 10 μM (pon10) was added at time of seeding to both R1 and R2 samples. A clear reduction in CFCs can be seen in both EV+pon10 and CC^{mut3} when compared with empty vector infection in R1 (Figure 4.5a). Importantly, clinical documentation of a T315I mutation is reported for R1. However, when R2 is subjected to the same treatment, little to no change can be seen between treatment groups in CFC number (Figure 4.5b). Because no difference was seen in CFCs, a growth assay was performed similar to that depicted in Figure 4.4a. This cell growth study depicts that cells maintain constant growth when

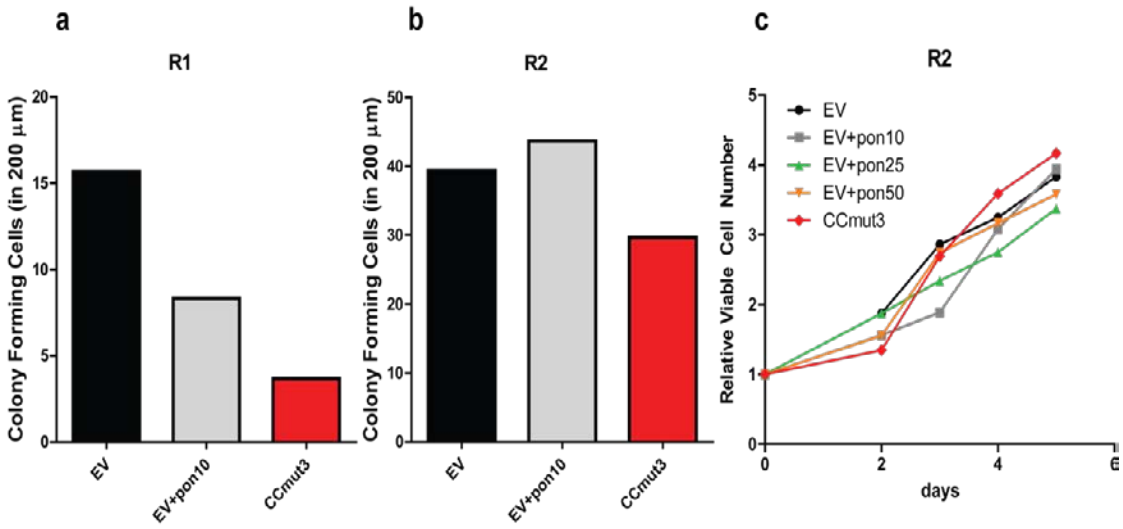

Figure 4.5. CC^{mut3} is effective in T315I accelerated but not blast crisis stage disease for one case ex vivo. Colony forming assays were set up with two samples from the same patient approximately 6 months between peripheral blood collections. Each sample was thawed and transduced with either EV or CC^{mut3}, following selection for transaction each sample was plated in triplicate (a) R1 displays sensitivity to transduction with CC^{mut3} or an intermediate dose of ponatinib (10 µM) compared to the EV transduced control. (b) R2 is a sample from the same individual in blast crisis who was reported to have failed ponatinib therapy at this time. CC^{mut3} here represents a ~25% reduction in colony forming cells. However, (c) growth analysis, also in a technical triplicate reveals little to no difference between increasing ponatinib does, the EV control or CC^{mut3}.

infected with EV (Figure 4.5c, circles), and are not markedly growth-inhibited following escalating doses of ponatinib (Figure 4.5c, squares; upright triangles; inverted triangles). Additionally, CC^{mut3} does not limit the growth of R2 compared with EV or EV+pon groups (Figure 4.5c, diamonds).

Discussion

TKIs have a proven track record in CML with over a decade of use producing significant clinical benefit (34). However, current challenges in CML therapy include mutational escape, BCR-ABL1-independent disease, and drug tolerance to name a few (35-39). BCR-ABL1-dependent resistance is often the product of acquired mutations following therapy, but can also be derived from unpressured selection for a more highly active BCR-ABL1 kinase in ND CML (12, 40, 41). Because kinase domain-targeted small molecule inhibitors will inherently be subject to acquired resistance arising from mutations in this domain, we chose to examine the ability of a designed coiled-coil dimerization domain inhibitor to disrupt the key event necessary for autophosphorylation of ABL1, oligomerization (42). We previously reported that transfection of the CC^{mut3} in unmutated BCR-ABL1-expressing K562 cells successfully limited BCR-ABL1-driven oncogenic activity (24). The activity of the CC^{mut3} in BCR-ABL1 mutants was evaluated in this study.

First we verified that the CC^{mut3} did not cause significant toxicity in cells that are BCR-ABL1-null. We completed this objective by measuring the induction of apoptosis, proliferation, and generation of CFCs in a methylcellulose colony forming assay in the IL-3-dependent Ba/F3 parental cell line. In all experiments we see no difference between

transfection with an empty vector expressing mCherry and the same vector with CC^{mut3} inserted on the C-terminus of mCherry as a fusion protein (Figure 4.1a, 2a, and 3a). These observations are in agreement with previous studies in BCR-ABL1-null lines COS-7 and 1471.1 (43), but these experiments represent an important control in a cell line with hematologic origin. Specifically, a low level of apoptotic cells is observed (mean of 5.97% for EV vs. 6.70% for CC^{mut3}) with the majority of the cells falling in the live event gate (mean of 75.1% for EV vs. 66.6% for CC^{mut3}, not significantly different by unpaired t-test).

Since previous studies demonstrated that CC^{mut3} was effective in limiting proliferation and transformation, as well as inducing caspase-dependent apoptosis in p210-BCR-ABL1 expressing human-origin K562 cells, we proceeded to validate efficacy of this construct in the murine pro-B cell line Ba/F3 with stably expressed p210 BCR-ABL1 (with concurrent expression of GFP as an indicator of transduction), conferring IL-3 independence (44). In our studies using this cell line, we found that CC^{mut3} was most effective in reducing the oncogenic transformation, as measured by CFCs, and almost completely eliminated CFCs in the same area compared with EV control (Figure 4.3b). Significant differences were also seen at 96 h in the proliferation assay (Figure 4.2b), and in apoptosis induction (Figure 4.1b). These results are in accordance with our previous report of the CC^{mut3} in p210 BCR-ABL1 leukemia cells (24).

Mutational status of BCR-ABL1 is an important factor in the choice of treatment for CML. Some patients with later stage disease became imatinib-resistant after initially responding to therapy. The cause of this resistance is typically due to a single mutation in the BCR-ABL1 kinase domain (45). Second generation inhibitors are able to treat some

but not all mutant BCR-ABL1, but the third-generation pan-BCR-ABL1 inhibitor, ponatinib, has demonstrated impressive potency against all known mutants (46). However, clinical recommendations still stress the importance of enrollment in trials for new therapies to circumvent problems such as drug intolerance, BCR-ABL1-independent resistance, and potential new acquired resistance to newer therapies (e.g., ponatinib) (39, 47). With this in mind, we chose to examine the efficacy of CC^{mut3} in BCR-ABL1 mutant cell lines. We first chose the single mutant E255V for analysis because of varying documented activity against this mutant with current TKIs as discussed previously here and elsewhere (48).

The retrovirally-transduced, and p210 BCR-ABL1-containing, Ba/F3 cell line is conducive to high-throughput mutational analysis, and has been increasingly used as a model for drug screening (49). Efficacy of CC^{mut3} in cells harboring the E255V mutant was greater than or equal to that seen in the p210 wild-type strain. Apoptosis induction represented a more than 4-fold increase when treated with CC^{mut3} compared with EV (Figure 4.1c), while proliferation capacity was reduced by CC^{mut3} at both 72 and 96 h time points following transfection (Figure 4.2c). On the background of the E255V mutation, the CC^{mut3} appears to outperform the antileukemic activity seen in p210 cells, though direct statistical comparisons are not made here. Transformative ability is similarly inhibited by CC^{mut3} in wild-type and E255V experiments (Figure 4.3b and c).

While varying TKI inhibitory potency is observed against many single kinase mutants, the T315I gatekeeper mutation was universally out of reach until the development of ponatinib, designed with that mutation in mind (50). Though ponatinib is now approved for TKI-resistant patients and indicated specifically for individuals with

the T315I mutation, it was necessary to examine CC^{mut3} efficacy in the context of this single mutant. We found that the CC^{mut3} was able to induce a significant level of apoptosis compared to EV control in T315I cells (Figure 4.1e). Additionally, significant differences were observed in cell proliferation in cells with the T315I mutation at both 72 and 96 h (Figure 4.2e) following CC^{mut3} transfection. Finally, CC^{mut3} significantly reduced CFCs compared to EV control (Figure 4.3e). Taken together, these data support the utility of CC^{mut3} efficacy against the T315I kinase domain mutant.

Third generation TKIs including DCC-2036 (currently in development) and ponatinib clearly show inhibition of kinase activity even in the presence of the T315I mutation (51). However, clinical efficacy following the genesis of compound mutants has not been evaluated. Recently, a study was completed by the Deininger group demonstrating the impaired utility of both ponatinib and DCC-2036 when used in the Ba/F3 BCR-$ABL1^{E255V/T315I}$ compound mutant cell line. The IC_{50} was increased from 2.3 nM in nonmutant background to 425 nM, or 27 nM to 1272 nM in this compound mutant line for ponatinib and DCC-2036, respectively (52). Because of these observations, we chose to evaluate the efficacy of CC^{mut3} in the context of this compound mutant.

CC^{mut3} demonstrated efficacy, yet potency varied following treatment of compound mutant cells when compared to single mutants alone. Apoptotic induction in compound mutant cells was significantly more than the EV control (Figure 4.1e), yet not as significant as either mutant alone (compare Figure 4.1c and d). However, no significant difference in apoptosis was measured between single mutants and the compound mutant, all of which were treated with CC^{mut3} (one-way ANOVA, Tukey's posttest). On the other hand, proliferation as measured by cell metabolism (Figure 4.2)

shows a very small, yet significant difference at 96 h between EV and CC^{mut3} treated compound mutant cells (Figure 4.2e). However, when compared with the values for wild-type, single and compound mutants, CC^{mut3}-treated compound mutant cells do not demonstrate a significant shift from the Ba/F3 parental control cells (one-way ANOVA, Tukey's posttest, $p<0.001$). When we examine the transformation potential of the compound mutant line, treatment with CC^{mut3} confers a significant reduction in CFCs when compared with EV control (Figure 4.3e). However, when CC^{mut3} treatments are compared between cell lines the potency appears reduced compared with other lines (Figure 4.3e vs. 3b, c, and d). In fact, the compound mutant is different than all other BCR-ABL1 expressing lines measured (one-way ANOVA, Tukey's posttest, $p<0.05$), but is also different from the Ba/F3 null line ($p<0.001$).

Reviewing the data in the Ba/F3 cell lines demonstrates the ability of CC^{mut3} to exert a notable inhibition in BCR-ABL1 driven activity. This is achieved with little toxicity to BCR-ABL1 null lines. Furthermore the ability of CC^{mut3} to maintain this activity in the presence of single and in some cases compound mutant versions of BCR-ABL1 is an important step forward for a single-agent selective inhibitor of BCR-ABL1.

We went on to test the efficacy of CC^{mut3} delivered using a lentivirus in primary CML patient samples. In the samples selected, CC^{mut3} matched the performance of the most relevant TKI used for these conditions. Following lentiviral infection and selection of infected cells, primary cells from newly diagnosed CP-CML patients exhibited a sensitivity to CC^{mut3} and IM when compared to EV control in both a cell growth assay (Figure 4.4a) and methylcellulose colony forming assays performed on four ND CP-CML samples (Figures 4b-f). Significant inhibition was seen demonstrating efficacy in a

relevant clinical model.

Next, we sought to evaluate the activity of CC^{mut3} in a primary sample with a T315I mutation. Although not initially noted during sample selection, the two samples that were evaluated (R1 and R2) came from the same patient at two different time points. R1 was during accelerated disease phase with a documented T315I mutation. The current course of therapy for this individual at time of sample collection was 500 mg hydrea b.i.d., 0.5 mg anagrelide b.i.d., and intermittent peg-interferon. A second sample (R2) was collected when the patient presented in distress at a local hospital; they were taking ponatinib at the time of collection but had progressed to blast crisis phase. These samples were separately infected with lentiviral constructs for EV or CC^{mut3} and evaluated for colony formation in methylcellulose 14 days after cell sorting. Treatment with 10 nM ponatinib or with CC^{mut3} reduced the number of CFCs compared to EV control in R1 (Figure 4.5a). However, a demonstrated reduction in colony number following treatment with any agent could not be seen in R2 (Figure 4.5b). To evaluate any potential susceptibility to ponatinib or CC^{mut3}, a cell growth assay was started, and monitored for 5 days. Again, no observable difference was seen in this assay between any constructs (Figure 4.5c). Following this result, we examined the status of p-Abl and p-CrkL in these samples and discovered no change in status of these phosphorylated proteins between untreated and ponatinib or CC^{mut3} treated samples, indicating the sample is not BCR-ABL1-independent (data not shown). Though no specific cytogenetic data for the R2 samples is available, we may speculate that secondary genetic or molecular abnormalities are contributing to the lack of efficacy in this CML blast crisis individual sample. Previously reported secondary abnormalities include p53 mutations (25-30%), p16/ARF

mutations (50%), or amplification of BCR-ABL1 (38%) (53).

In summary, novel therapeutics that selectively target BCR-ABL1 outside the kinase domain do not currently exist in the clinical domain. We have previously shown efficacy of such an inhibitor which is directed to disrupt the oligomerization event necessary for kinase activity of BCR-ABL1. This report demonstrates activity in another hematopoietic line with BCR-ABL1, in addition to minimal toxicity in BCR-ABL1 null status. Additionally, efficacy in single-mutant BCR-ABL1 cells is evident, where the majority of kinase inhibitors are not able to treat these single-mutants. Moreover, CC^{mut3} appears to have some activity in against compound-mutants, where current state-of-the art inhibitors struggle. Finally, we show the effectiveness of CC^{mut3} in treating newly diagnosed CP-CML and accelerated phase-CML with T315I mutations ex vivo. However, when cells become BCR-ABL1-independent, CC^{mut3}, or any single-agent BCR-ABL1 directed therapeutic fails to produce a response.

Going forward we plan to improve the delivery method of CC^{mut3}, moving away from lentiviral delivery and toward a cell-penetrating peptide. Finally, stapled peptides will be explored to increase stability and potency. While all of these tasks are aimed at better BCR-ABL1 targeting, BCR-ABL1-independent targets could be incorporated by using peptide-drug conjugates in future studies.

Author Contributions

DWW designed and preformed the research, collected data, analyzed and interpreted data, and wrote the manuscript; AME assisted with patient sample experiments; BJB and GDM assisted with cell line experiments; MWD supported the

research on patient samples and provided the cell lines; CSL supported the research and edited the manuscript.

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Keywords

CML, BCR-ABL1, Coiled-coil, TKI-resistant, CC^{mut3}, kinase mutation, compound mutant

References

1. Nowell PC. The minute chromosome (phl) in chronic granulocytic leukemia. *Blut.* 1962;8(65-6.

2. Bartram CR, de Klein A, Hagemeijer A, van Agthoven T, Geurts van Kessel A, Bootsma D, Grosveld G, Ferguson-Smith MA, Davies T, Stone M, et al. Translocation of c-ab1 oncogene correlates with the presence of a philadelphia chromosome in chronic myelocytic leukaemia. *Nature.* 1983;306(5940):277-80.

3. Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, Zimmermann J, and Lydon NB. Effects of a selective inhibitor of the abl tyrosine kinase on the growth of bcr-abl positive cells. *Nat Med.* 1996;2(5):561-6.

4. Naldini L, Stacchini A, Cirillo DM, Aglietta M, Gavosto F, and Comoglio PM. Phosphotyrosine antibodies identify the p210c-abl tyrosine kinase and proteins phosphorylated on tyrosine in human chronic myelogenous leukemia cells. *Mol Cell Biol.* 1986;6(5):1803-11.

5. Evans JP, Wickremasinghe RG, and Hoffbrand AV. Tyrosine protein kinase substrates in philadelphia-positive human chronic granulocytic leukemia derived cell lines (k562 and bv173): Detection by using an immunoblotting technique. *Leukemia.* 1987;1(6):524-5.

6. Hanfstein B, Muller MC, Hehlmann R, Erben P, Lauseker M, Fabarius A, Schnittger S, Haferlach C, Gohring G, Proetel U, et al. Early molecular and cytogenetic response is predictive for long-term progression-free and overall survival in chronic myeloid leukemia (cml). *Leukemia.* 2012;26(9):2096-102.

7. Sawyers CL, Hochhaus A, Feldman E, Goldman JM, Miller CB, Ottmann OG, Schiffer CA, Talpaz M, Guilhot F, Deininger MW, et al. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: Results of a phase ii study. *Blood.* 2002;99(10):3530-9.

8. Hochhaus A, Kantarjian HM, Baccarani M, Lipton JH, Apperley JF, Druker BJ, Facon T, Goldberg SL, Cervantes F, Niederwieser D, et al. Dasatinib induces notable hematologic and cytogenetic responses in chronic-phase chronic myeloid leukemia after failure of imatinib therapy. *Blood.* 2007;109(6):2303-9.

9. le Coutre P, Ottmann OG, Giles F, Kim DW, Cortes J, Gattermann N, Apperley JF, Larson RA, Abruzzese E, O'Brien SG, et al. Nilotinib (formerly amn107), a highly selective bcr-abl tyrosine kinase inhibitor, is active in patients with imatinib-resistant or -intolerant accelerated-phase chronic myelogenous leukemia. *Blood.* 2008;111(4):1834-9.

10. Hehlmann R, Heimpel H, Hasford J, Kolb HJ, Pralle H, Hossfeld DK, Queisser W, Loffler H, Heinze B, Georgii A, et al. Randomized comparison of busulfan

and hydroxyurea in chronic myelogenous leukemia: Prolongation of survival by hydroxyurea. The german cml study group. *Blood.* 1993;82(2):398-407.

11. Ohnishi K, Ohno R, Tomonaga M, Kamada N, Onozawa K, Kuramoto A, Dohy H, Mizoguchi H, Miyawaki S, Tsubaki K, et al. A randomized trial comparing interferon-alpha with busulfan for newly diagnosed chronic myelogenous leukemia in chronic phase. *Blood.* 1995;86(3):906-16.

12. Branford S, Rudzki Z, Walsh S, Parkinson I, Grigg A, Szer J, Taylor K, Herrmann R, Seymour JF, Arthur C, et al. Detection of bcr-abl mutations in patients with cml treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the atp phosphate-binding loop (p-loop) are associated with a poor prognosis. *Blood.* 2003;102(1):276-83.

13. Cassuto O, Dufies M, Jacquel A, Robert G, Ginet C, Dubois A, Hamouda A, Puissant A, Luciano F, Karsenti JM, et al. All tyrosine kinase inhibitor-resistant chronic myelogenous cells are highly sensitive to ponatinib. *Oncotarget.* 2012;3(12):1557-65.

14. Burke AC, Swords RT, Kelly K, and Giles FJ. Current status of agents active against the t315i chronic myeloid leukemia phenotype. *Expert Opin Emerg Drugs.* 2011;16(1):85-103.

15. O'Brien S, Berman E, Moore JO, Pinilla-Ibarz J, Radich JP, Shami PJ, Smith BD, Snyder DS, Sundar HM, Talpaz M, et al. Nccn task force report: Tyrosine kinase inhibitor therapy selection in the management of patients with chronic myelogenous leukemia. *J Natl Compr Canc Netw.* 2011;9(S2):S1-25.

16. Mian AA, Schull M, Zhao Z, Oancea C, Hundertmark A, Beissert T, Ottmann OG, and Ruthardt M. The gatekeeper mutation t315i confers resistance against small molecules by increasing or restoring the abl-kinase activity accompanied by aberrant transphosphorylation of endogenous bcr, even in loss-of-function mutants of bcr/abl. *Leukemia.* 2009;23(9):1614-21.

17. Deininger M, Cortes, JE, Kim, D, Nicolini, FE, Talpaz, M, Baccarani, M, Müller, MC, Lustgarten, S, Rivera, VM, Clackson, T, Turner, CD, Haluska, FG, Guilhot, F, Hochhaus, A, Goldman, JM, Shah, NP, Kantarjian, HM, Hughes, TP. Impact of baseline mutations on response to ponatinib and end of treatment mutation analysis in patients with chronic myeloid leukemia. *J Clin Oncol 2013;31(Suppl):Abstract 7001.* 2013.

18. Khorashad JS, Kelley TW, Szankasi P, Mason CC, Soverini S, Adrian LT, Eide CA, Zabriskie MS, Lange T, Estrada JC, et al. Bcr-abl1 compound mutations in tyrosine kinase inhibitor-resistant cml: Frequency and clonal relationships. *Blood.* 2013;121(3):489-98.

19. Zhang J, Yang PL, and Gray NS. Targeting cancer with small molecule kinase inhibitors. *Nat Rev Cancer.* 2009;9(1):28-39.

20. Beissert T, Puccetti E, Bianchini A, Guller S, Boehrer S, Hoelzer D, Ottmann OG, Nervi C, and Ruthardt M. Targeting of the n-terminal coiled coil oligomerization interface of bcr interferes with the transformation potential of bcr-abl and increases sensitivity to sti571. *Blood.* 2003;102(8):2985-93.

21. Beissert T, Hundertmark A, Kaburova V, Travaglini L, Mian AA, Nervi C, and Ruthardt M. Targeting of the n-terminal coiled coil oligomerization interface by a helix-2 peptide inhibits unmutated and imatinib-resistant bcr/abl. *Int J Cancer.* 2008;122(12):2744-52.

22. Mian AA, Oancea C, Zhao Z, Ottmann OG, and Ruthardt M. Oligomerization inhibition, combined with allosteric inhibition, abrogates the transformation potential of t315i-positive bcr/abl. *Leukemia.* 2009;23(12):2242-7.

23. Dixon AS, Pendley SS, Bruno BJ, Woessner DW, Shimpi AA, Cheatham TE, 3rd, and Lim CS. Disruption of bcr-abl coiled coil oligomerization by design. *J Biol Chem.* 2011;286(31):27751-60.

24. Dixon AS, Miller GD, Bruno BJ, Constance JE, Woessner DW, Fidler TP, Robertson JC, Cheatham TE, 3rd, and Lim CS. Improved coiled-coil design enhances interaction with bcr-abl and induces apoptosis. *Mol Pharm.* 2012;9(1):187-95.

25. Daley GQ, Van Etten RA, and Baltimore D. Blast crisis in a murine model of chronic myelogenous leukemia. *Proc Natl Acad Sci USA.* 1991;88(24):11335-8.

26. La Rosee P, Corbin AS, Stoffregen EP, Deininger MW, and Druker BJ. Activity of the bcr-abl kinase inhibitor pd180970 against clinically relevant bcr-abl isoforms that cause resistance to imatinib mesylate (gleevec, sti571). *Cancer Res.* 2002;62(24):7149-53.

27. O'Hare T, Pollock R, Stoffregen EP, Keats JA, Abdullah OM, Moseson EM, Rivera VM, Tang H, Metcalf CA, 3rd, Bohacek RS, et al. Inhibition of wild-type and mutant bcr-abl by ap23464, a potent atp-based oncogenic protein kinase inhibitor: Implications for cml. *Blood.* 2004;104(8):2532-9.

28. Dexter TM, Garland J, Scott D, Scolnick E, and Metcalf D. Growth of factor-dependent hemopoietic precursor cell lines. *J Exp Med.* 1980;152(4):1036-47.

29. Woessner DW, and Lim CS. Disrupting bcr-abl in combination with secondary leukemia-specific pathways in cml cells leads to enhanced apoptosis and decreased proliferation. *Mol Pharm.* 2013;10(1):270-7.

30. Fleischman AG, Aichberger KJ, Luty SB, Bumm TG, Petersen CL, Doratotaj S, Vasudevan KB, LaTocha DH, Yang F, Press RD, et al. Tnfalpha facilitates clonal expansion of jak2v617f positive cells in myeloproliferative neoplasms. *Blood.* 2011;118(24):6392-8.

31. Schmid I, Krall WJ, Uittenbogaart CH, Braun J, and Giorgi JV. Dead cell discrimination with 7-amino-actinomycin d in combination with dual color immunofluorescence in single laser flow cytometry. *Cytometry.* 1992;13(2):204-8.

32. Vermes I, Haanen C, Steffens-Nakken H, and Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin v. *J Immunol Methods.* 1995;184(1):39-51.

33. Berridge MV, and Tan AS. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (mtt): Subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in mtt reduction. *Arch Biochem Biophys.* 1993;303(2):474-82.

34. Jabbour E, and Kantarjian H. Chronic myeloid leukemia: 2012 update on diagnosis, monitoring, and management. *Am J Hematol.* 2012;87(11):1037-45.

35. Koren-Michowitz M, le Coutre P, Duyster J, Scheid C, Panayiotidis P, Prejzner W, Rowe JM, Schwarz M, Goldschmidt N, and Nagler A. Activity and tolerability of nilotinib: A retrospective multicenter analysis of chronic myeloid leukemia patients who are imatinib resistant or intolerant. *Cancer.* 2010;116(19):4564-72.

36. Jabbour E, Hochhaus A, Cortes J, La Rosee P, and Kantarjian HM. Choosing the best treatment strategy for chronic myeloid leukemia patients resistant to imatinib: Weighing the efficacy and safety of individual drugs with bcr-abl mutations and patient history. *Leukemia.* 2010;24(1):6-12.

37. Hehlmann R, Lauseker M, Jung-Munkwitz S, Leitner A, Muller MC, Pletsch N, Proetel U, Haferlach C, Schlegelberger B, Balleisen L, et al. Tolerability-adapted imatinib 800 mg/d versus 400 mg/d versus 400 mg/d plus interferon-alpha in newly diagnosed chronic myeloid leukemia. *J Clin Oncol.* 2011;29(12):1634-42.

38. O'Hare T, Eide CA, and Deininger MW. Bcr-abl kinase domain mutations, drug resistance, and the road to a cure for chronic myeloid leukemia. *Blood.* 2007;110(7):2242-9.

39. Woessner DW, Lim CS, and Deininger MW. Development of an effective therapy for chronic myelogenous leukemia. *Cancer J.* 2011;17(6):477-86.

40. Carella AM, Garuti A, Cirmena G, Catania G, Rocco I, Palermo C, Pica G, Pierri I, Miglino M, Ballestrero A, et al. Kinase domain mutations of bcr-abl identified at diagnosis before imatinib-based therapy are associated with progression in patients with high sokal risk chronic phase chronic myeloid leukemia. *Leuk Lymphoma.* 2010;51(2):275-8.

41. Klamova H, Faber E, Zackova D, Markova M, Voglova J, Cmunt E, Novakova L, Machova-Polakova K, Moravcova J, Dvorakova D, et al. Dasatinib in imatinib-resistant or -intolerant cml patients: Data from the clinical practice of 6 hematological centers in the czech republic. *Neoplasma.* 2010;57(4):355-9.

42. McWhirter JR, Galasso DL, and Wang JY. A coiled-coil oligomerization domain of bcr is essential for the transforming function of bcr-abl oncoproteins. *Mol Cell Biol.* 1993;13(12):7587-95.

43. Constance JE, Woessner DW, Matissek KJ, Mossalam M, and Lim CS. Enhanced and selective killing of chronic myelogenous leukemia cells with an engineered bcr-abl binding protein and imatinib. *Mol Pharm.* 2012;9(11):3318-29.

44. Ahmed M, Dusanter-Fourt I, Bernard M, Mayeux P, Hawley RG, Bennardo T, Novault S, Bonnet ML, Gisselbrecht S, Varet B, et al. Bcr-abl and constitutively active erythropoietin receptor (cepor) activate distinct mechanisms for growth factor-independence and inhibition of apoptosis in ba/f3 cell line. *Oncogene.* 1998;16(4):489-96.

45. McCormick F. New-age drug meets resistance. *Nature.* 2001;412(6844):281-2.

46. Dufies M, Cassuto O, Jacquel A, Robert G, and Auberger P. Ponatinib circumvents all types of imatinib resistance in chronic myelogenous leukemia cell lines. *Cell Cycle.* 2013;12(11):1645-6.

47. Jain P, Kantarjian H, and Cortes J. Chronic myeloid leukemia: Overview of new agents and comparative analysis. *Curr Treat Options Oncol.* 2013;14(2):127-43.

48. Bradeen HA, Eide CA, O'Hare T, Johnson KJ, Willis SG, Lee FY, Druker BJ, and Deininger MW. Comparison of imatinib mesylate, dasatinib (bms-354825), and nilotinib (amn107) in an n-ethyl-n-nitrosourea (enu)-based mutagenesis screen: High efficacy of drug combinations. *Blood.* 2006;108(7):2332-8.

49. Warmuth M, Kim S, Gu X-j, Xia G, and Adrián F. Ba/f3 cells and their use in kinase drug discovery. *Current Opinion in Oncology.* 2007;19(1):55-60 10.1097/CCO.0b013e328011a25f.

50. Zhou T, Commodore L, Huang WS, Wang Y, Thomas M, Keats J, Xu Q, Rivera VM, Shakespeare WC, Clackson T, et al. Structural mechanism of the pan-bcr-abl inhibitor ponatinib (ap24534): Lessons for overcoming kinase inhibitor resistance. *Chem Biol Drug Des.* 2011;77(1):1-11.

51. Chan WW, Wise SC, Kaufman MD, Ahn YM, Ensinger CL, Haack T, Hood MM, Jones J, Lord JW, Lu WP, et al. Conformational control inhibition of the bcr-abl1 tyrosine kinase, including the gatekeeper t315i mutant, by the switch-control inhibitor dcc-2036. *Cancer Cell.* 2011;19(4):556-68.

52. Eide CA, Zabriskie MS, Adrian LT, Lange T, Deininger MW, Druker BJ, and O'Hare T. Resistance profiling of bcr-abl compound mutations linked to tyrosine kinase inhibitor therapy failure in chronic myeloid leukemia. *ASH Annual Meeting Abstracts.* 2011;118(21):1416-.

53. Calabretta B, and Perrotti D. The biology of cml blast crisis. *Blood.* 2004;103(11):4010-22.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Targeting a New Domain for Selective BCR-ABL1 Inhibition

Summary

The discovery of the Philadelphia (Ph) chromosome in 1960 linked the BCR-ABL1 reciprocal translocation to a Ph-positive myeloproliferative disorder (1-3). Chronic myeloid leukemia (CML) is universally caused by this chromosomal rearrangement, but the BCR-ABL1 reciprocal translocation can also be found in 25-30% of acute lymphoblastic leukemias (ALL) (4-6). For more than a decade, small molecule tyrosine kinase inhibitors have changed the course of Ph^+ disease management, transforming a fatal disease into a manageable chronic condition, most notably in CML. This success was accomplished by inhibiting activity of the BCR-ABL1 constitutively active tyrosine kinase, the central event in disease initiation, first with imatinib, and later with subsequently improved TKIs. BCR-ABL1-selective inhibitors have focused entirely on the Y-kinase domain, with the exception of a series of investigations by Ruthart and colleagues, which proposed a competitive binder for the essential upstream BCR-ABL1 dimerization/oligomerization event (7). Over the last 5 years, the Lim laboratory has focused on the N-terminal domain of BCR-ABL1, specifically the coiled-coil (CC), for potential therapeutic intervention in Ph^+ disease. Our studies depart from the Ruthardt

et al., work by not using a wild-type unmodified section of the CC, but instead are based on rationally designed mutations which facilitate preferential binding of the "CC^{mut}" to BCR-ABL1 and limiting interactions with itself (also called homooligomerization). This increased heteroligomerization specificity utilized by CC^{mut} peptides significantly shifts the preference for binding to (and inhibition of) the CC in BCR-ABL1 compared to homoligomerization between multiple BCR-ABL1 CC domains.

The studies presented here focus on the ability of our therapeutic to limit BCR-ABL1 leukemogenesis in general; however, specific attention is given to the main barriers to the universal success of TKIs – resistance. A review of current therapy presented in Chapter 2 discussed the milestones of historical therapies and those under development, but also articluated necessary steps to cure CML. The third chapter explored using the CC^{mut2} as a baseline therapy and the possible avenues for adding secondary agents with the hope of addressing minimum residual disease, or BCR-ABL1-independence. The most recent series of studies, presented in Chapter 4, investigated the potential to overcome mutational escape in the Y-kinase domain. Key points from these chapters will be reviewed and discussed here in reverse order.

BCR-ABL1-Dependent Resistance: Solving TKI-Resistance Through Oligomeric Disruption of BCR-ABL

Oncogenic inhibition across varying mutational status of BCR-ABL1 can be achieved by introduction of the CC^{mut3}. We observed that cells without BCR-ABL1 did not exhibit significant toxic effects following exposure to CC^{mut3}, and that levels we did see were not different than those exposed to a control construct delivered using the same

mechanism. Accordingly, we showed p210-BCR-ABL1 is sensitive to CC^{mut3} administration in the Ba/F3 cell line. This is in agreement with previous work by our group in K562 cells (8-10).

Kinase mutants are of increasing importance for CML disease management, with only one FDA approved agent available to treat all known single kinase domain mutants. We examined the efficacy of CC^{mut3} in two single Y-kinase domain mutants. E255V is a p-Loop mutation that confers resistance to imatinib, and a significant therapeutic barrier (requiring dose-escalation) for nilotinib and dasatinib. T315I is only treatable with the pan-BCR-ABL1 inhibitor ponatinib (FDA approved with an indication for patients who are TKI-resistant or have T315I), or the "switch-pocket" inhibitor DCC-2036 (which recently completed a Phase I/II trial) (11, 12). We observed a dramatic induction of apoptosis and reduction in proliferation and transformation following treatment with CC^{mut3} compared to EV control in both single BCR-ABL1 mutant cell lines. Finally, the compound mutant E255V/T315I was tested. This compound mutant was found to significantly impair the potency (measured by IC_{50}) of both ponatinib and DCC-2036 (13). CC^{mut3} demonstrated significant induction of apoptosis, as well as limiting colony forming ability with this compound mutant. Though we also noted a reduction in proliferation, this effect was less dramatic, yet still significant when compared with wild-type or single mutants of BCR-ABL1 in Ba/F3 cells.

Though it is difficult to compare across cell lines, owing to possible differences in kinase activity (14) and growth potential of the cells prior to setting up assays, the data for CC^{mut3} across all Ba/F3 cell lines is presented side-by-side and normalized to the EV control (Figure 5.1). When viewed in this manner, single kinase mutants retain sensitivity

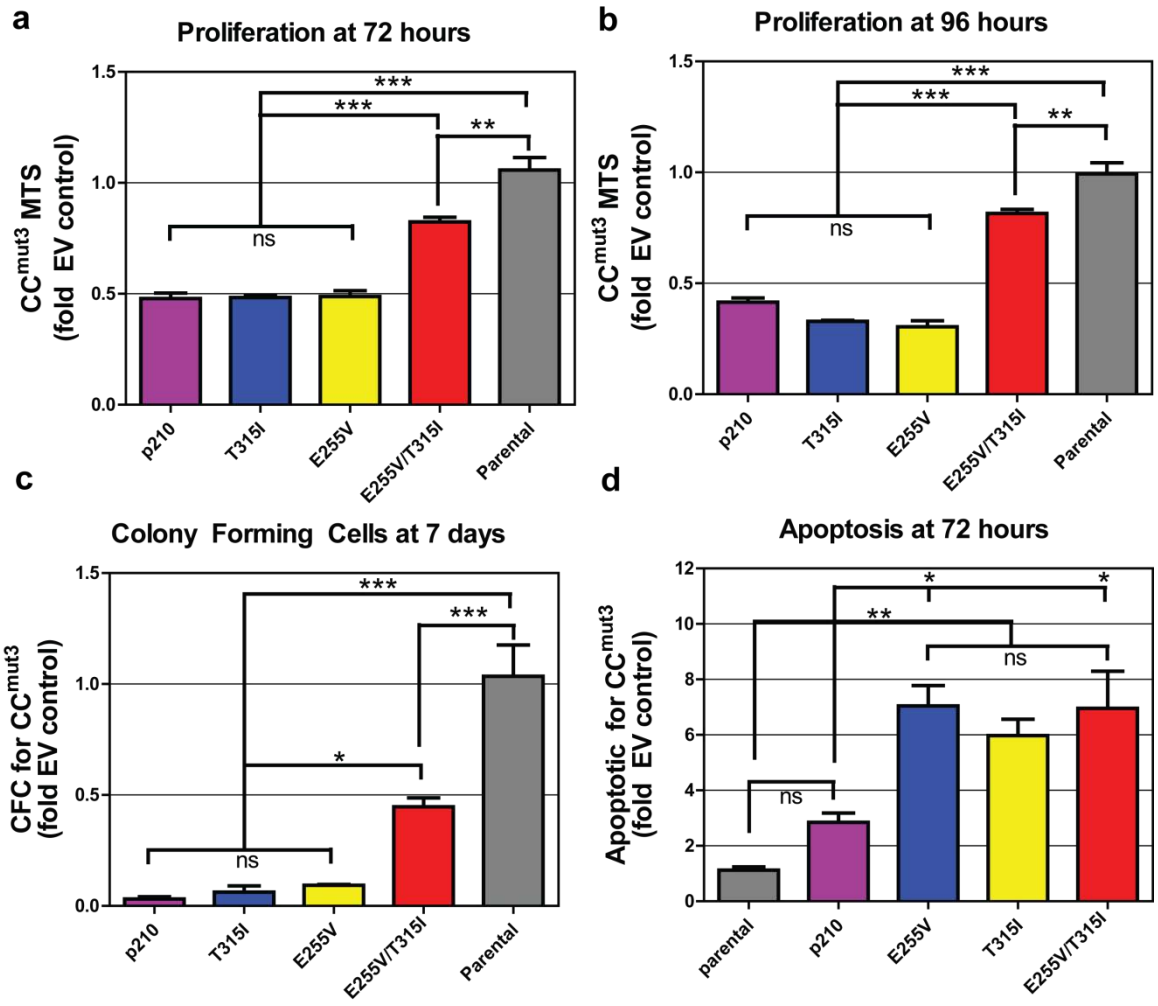

Figure 5.1 Summary of Ba/F3 CC^{mut3} experiments from Chapter 4. EV constructs are omitted from this data analysis for direct comparison of CC^{mut3} activity across varying cell line backgrounds. Initial comparisons were made only between EV and CC^{mut3} for each cell line. (a) MTS assays at 72 and (b) 96 h are shown across all cell lines for comparison. (c) Colony forming assay after one week in methylcellulose culture. (d) Apoptosis assay conducted after 72 h. n=3. One-way ANOVA with Tukey's posttest, ns, not significant; *p<0.05, **0.01, ***p<0.001.

to CC^{mut3} while the compound-mutant stands out as being less sensitive, though still yielding a significant reduction (Figure 5.1c). CC^{mut3} apoptotic activity in p210-containing cells is limited for this specific experiment, which becomes evident when comparing p210 to parental Ba/F3 controls (Figure 5.1d).

Fortunately, we were able to access patient samples for later experiments. These samples provided an advanced model for testing the efficacy of CC^{mut3}, and an important step forward in efficacy validation. Newly diagnosed patients were sensitive to CC^{mut3} ex vivo as measured by cell growth and colony formation. Interestingly, a T315I mutant was also sensitive to CC^{mut3} in accelerated disease phase, but not in blast crisis phase. We are currently attempting to verify that this sample is BCR-ABL1-independent through analysis of phospho-ABL1 and the specific substrate for ABL1, phospho-CrkL.

While we did see activity in the T315I mutant of BCR-ABL1 with the CC^{mut3}, Ruthardt and colleagues did not observe a potent reduction in transformation following treatment with the CC helix 2 construct. They did, however observe phospho-ABL reduction (15). This is a discrepancy that may be explained by either our incorporation of mutations or the inclusion of the helix 1 portion of CC. However, we have not yet completed a phospho-protein analysis of the ABL1 signaling network following treatment with CC^{mut3} in a mutant BCR-ABL1 background.

BCR-ABL1-Independent Resistance: Necessity of a Second Leukemia-Specific Inhibitory Agent

Recent reports validate the survival of cells which harbor BCR-ABL1, but are not dependent on its signaling (16). This state of BCR-ABL-independence confers a

persistent cell population, likely leukemic stem cells (LSCs) which may be quiescent, leading to a small population of cells that will not die following TKI treatment (17). Presence of these cells leads to an overwhelming majority of patients who must remain on TKI therapy chronically, though a small percentage are able to stop TKI therapy and remain disease-free (18, 19). With this in mind, we decided to investigate the activity of CC^{mut3} combined with one of three secondary agents which targeted disparate pathways not directly involved with BCR-ABL1, but specific to the LSC vs. a normal HSC.

While we did not directly evaluate the hypothesis of BCR-ABL1-independence, we did investigate the ability of secondary agents with or without CC^{mut2} to potentiate cell death and reduce proliferation in a blast crisis phase immortalized BCR-ABL1 cell line (K562). For this series of experiments we chose assays that had previously shown efficacy with CC^{mut2}. Depending on the assay, we saw differential specificity toward enhancement of antileukemic biology for the three agents. The most consistent response was to chloroquine (CQ), which at 10 μM was effective at blocking the autophagocytic process, leading to a combined effect of increased caspase 3/7 activity, drastically reduced proliferation and a somewhat nonspecific induction of apoptosis (as measured by AnnexinV and 7AAD). The nonspecific nature of apoptosis caused by CQ in this experiment may be the result of increased externalized phosphatidylserine mediated by the autophagic blockade and overproduction of membranous components inserted into the cell membrane. The mucin-1 peptide inhibitor developed by the Kufe group also showed promise, though further testing of a control protein revealed some significant toxicity (data not shown), which could be a product of the arginine-rich cell penetrating peptide on the N-terminus (20-23). The Alox5 pathway showed weak specificity toward

combinatorial reduction of colony formation. While interesting, several reports now show the role of arachadonic acid and Alox5 as differentiation controllers in leukemic stem cells (24, 25). Going forward, testing CC^{mut3} and Alox5 inhibitor combinations in LSCs may show increased differentiation markers such as CD11b/c and CD14 (20, 26).

Finally, the ability to study the true consequences of these combinations in a more robust model of BCR-ABL1-independence is warranted. Based on the data generated in primary patient samples (noted above), we would propose testing the BCR-ABL1 T315I blast crisis patient sample (R2) from Chapter 4 with a chloroquine combination (discussed in Chapter 3). Other interesting models would include a stromal-driven population of primary cells which would more closely model the BCR-ABL1-independent LSC which is most likely in the bone marrow microenvironment (27, 28).

Clinical Implications for Current and Future Therapies

Finally, it is necessary to update some information regarding available therapies, new developments concerning treatment strategies and other clinical concerns. Since the publication of Chapter 2 in 2011 (29), three new drugs have been approved by the FDA for CML therapy, the first of which, ponatinib, has already been mentioned elsewhere in this work. Accelerated approval for ponatinib was given in December 2012 under the trade name Iclusig™. Its specific indication is for:

> the treatment of adult patients with chronic phase, accelerated phase, or blast phase chronic myeloid leukemia (CML) that is resistant or intolerant to prior tyrosine kinase inhibitor therapy or Philadelphia chromosome positive acute lymphoblastic leukemia (Ph+ALL) that is resistant or intolerant to prior tyrosine kinase inhibitor therapy. (30)

Ponatinib does carry a black box warning for arterial thrombosis and hepatotoxicity, with

these adverse events occurring in 11% and three total patients, respectively (31). This potent pan-BCR-ABL1 inhibitor does effectively treat the T315I mutant as extensively described elsewhere in this thesis, and is the only approved drug indicated for these patients.

Bosutinib was granted approval in September of 2012 and was discussed in Chapter 2. Pfizer's contribution to the CML landscape is classified as a second-generation TKI, with similar indications as ponatinib. However, it does lack the ability to treat the T315I mutant.

Finally, omacetaxine mepesuccinate (Synribo™) is a recently-approved therapeutic which is also indicated for chronic or accelerated phase CML patients who have resistance or intolerance to two or more TKIs. Approval for the injectable omacetaxine mepesuccinate, a protein translation inhibitor, was granted in October 2012. Interestingly, omacetaxine mepesuccinate, also known as homoharringtonine, was initially identified more than 40 years ago in leukemia-related studies (32). However, further development was put on hold following the success of TKIs. Mirroring its indications, it is clear that this drug was put back on the development track to address TKI-resistance or potential BCR-ABL1-independence (33).

As the therapeutic landscape becomes increasingly crowded, with five TKIs and one nontargeted agent approved in the last twelve years, the clinical management of CML becomes more of a puzzle. While we generally discuss the TKIs as "targeted" to the ABL kinase domain, the truth is they are simply targeted to kinases in general, with varying activity and specificity depending on the agent (see Table 2.1) (29). The secondary goal of clinical care will shift toward managing drug side-effects, with the first priority of

stabilizing the disease state.

There is also increasing prognostic evidence that a short time to CCyR or MMR is predictive of a durable response. This can be aided with early diagnosis and treatment with potent drugs (34, 35). Additionally, a renewed effort to maintain an international scale for PCR detection of BCR-ABL1 from RQ-PCR with the hope that certified labs will standardize the values for BCR-ABL1 copy number, leading to a uniform clinical interpretations of disease state (36). Finally, there has been continued effort to evaluate the dose scheduling or discontinuation of TKIs. Most recently, a discontinuation study evaluated patients who had undetectable minimal residual disease and found 47% did not relapse (37). Almost simultaneously, a report was published detailing a one-month-on, one-month-off study of elderly patients with CML. Patients on this study may have shown enhanced molecular disease markers, but maintained an acceptable level of overall and progression-free survival (38). This is important particularly because of potential comorbidities in elderly patients and the ability to reduce dosing requirements of the drug.

In conclusion, though many new drugs are available for CML, there is still a need for innovative new therapeutics that display curative potential. The ability to circumvent either BCR-ABL1-dependent (mutational escape) and/or -independent resistance will allow this critical breakthrough in CML therapy. Moreover, any drug which can reduce significant off-target/adverse events will be promising.

Future Directions for Therapeutic Development

Introduction

Significant progress has been made since the inception of the CC^{mut} concept. The initial focus on the CC of BCR was to develop a capture motif that could be combined with a ligand-inducible protein switch (39). By harnessing the protein switch developed by the Lim lab, able to drag the BCR-ABL1 to the nucleus (40-42), we aimed to restore the protein-tyrosine kinase 1 (c-Abl) apoptotic activity to the cell afflicted with the BCR-ABL1 translocation and fusion products (43). To that end, the Lim laboratory attempted to modify the CC domain for better binding, resulting in the CC^{mut2} and later the CC^{mut3} (8, 9). Additionally, exploration of mitochondrial targeting of BCR-ABL1 was explored, which produced impressive cytotoxic effects especially with the addition of imatinib, ultimately aiding in releasing BCR-ABL1 from its actin fillament tether (10, 44).

This body of work demonstrates the efficacy of $CC^{mut2/3}$ as a combination therapy in concert with a secondary agent for enhanced cell toxicity, or alone against TKI-resistant cell lines harboring kinase mutations. Most importantly, we show efficacy in an advanced CML model, as close to the clinic as possible – including samples from patients with CML, and in a patient with TKI-resistance in accelerated phase. However, we fail to demonstrate efficacy in a sample from a patient in blast crisis phase disease, who is also resistant to ponatinib. In blast crisis phase CML, significant disease remodeling can occur (45), possibly generating BCR-ABL1-independence. With this in mind there are several questions which need answering and there are considerable opportunities for therapeutic advancement.

What Happens to BCR In the Context of CC^{mut3} Therapy?

Partners

As alluded to in Chapter 1, the function of p160 BCR in the greater context of normal cellular biology is not yet clear. To elucidate the potential unwanted interactions, a complete analysis of CC^{mut3}/BCR partners is needed. However, even with this information, we must rely on the scientific community at large to contribute to the overall knowledgebase. For example, the fact that BCR and ABR have a RAC/GAP function, which is important in neuronal biology, may have little consequence unless that process also requires oligomerization of BCR (46). Moreover, the CC^{mut3} would have to gain access to the neuronal compartment through the blood-brain barrier to have an effect on this function.

Notwithstanding, we envision a binding assay that begins with an affinity tagged (e.g., 6xHIS, Flag, HA or Myc) CC^{mut3}, which is then introduced into BCR-ABL1 null cells. Following expression and incubation, coimmunoprecipitation of the binding factors with CC^{mut3} would be carried out. This product could then be separated using SDS-PAGE, stained for protein, and bands excised for analysis and identification via mass spectroscopy. This process could be performed for as many cell lines as was necessary and even translated to primary cells isolated from patients.

BCR-Mediated Mutational Escape

A significant portion of this work was dedicated to discussing how CC^{mut3} is able to circumvent acquired kinase domain mutations in ABL1. However, the possibility that kinase-independent resistance to CC^{mut3} could arise is very real. Potential mechanisms are

somewhat limited, we contend, due to the inherent dimerization specification in the CC domain. To elaborate, we would argue that the purpose of the CC domain is to facilitate dimerization. CC^{mut3} has been designed to preferentially bind to the CC. It is possible that if a mutation occurred in this region, both CC^{mut3} and other wild-type CC (e.g., from BCR-ABL1 dimer partners) domains would fail to bind, resulting in an autoinactivation of oligomerization. This would ultimately have the same result as seen in deletion or binding disruption of the CC (47).

To test this hypothesis, we propose a mutagenesis screen to identify potential CC mutants that may confer resistance to CC^{mut3} and a simultaneous growth advantage. For this experiment, we would use Ba/F3 p210 cells and subject them to N-ethyl-N-nitrosourea (ENU)-based mutagenesis (48). Following mutagen exposure, cells would be transduced with CC^{mut3} virus with a selectable (hygromycin or similar) eukaryotic marker to ensure expression. Cells that continued to grow above baseline would be removed, and lysed and their DNA amplified. The CC domain, along with the Y-kinase domain for completeness, could then be sequenced using multiple reads to determine a consensus of CC^{mut3}-inactivating mutations. From this analysis we would hope to identify therapeutic barriers or susceptibility to CC-mediated mutational escape following administration of CC^{mut3}. However, it is conceivable that mutant CCs already exist either in BCR or BCR-ABL1 disease state. While we are not aware of any effort to analyze the sequence of CC patients to date, a comprehensive analysis of current domain mutations in primary samples could easily be performed as a companion to the ENU-mutagenesis screen in cells.

Multiple-Domain Targeting of BCR-ABL1

Targeted therapeutics directed to inhibit the Y-kinase domain of BCR-ABL1 have been extremely successful (49). It may come as a surprise, however, that Ruthardt et al., reported that coadministration of the α-2 helix and imatinib (targeting of CC and Y-kinase) results in enhanced sensitivity to the TKI (7). Recently Geoff Miller in the Lim lab explored a combination study treating cells with CC^{mut3} and with escalating concentrations of ponatinib. He discovered that ponatinib alone was able to significantly inhibit measured oncogenic processes, but coadministration of CC^{mut3} could elicit the same effect at a lower dose of ponatinib. A selection of that data is presented in Figure 5.2 (unpublished data). Results were similar in other assays with a significant dose-lowering capability at 1 nM ponatinib or 10 nM in T315I mutant cells.

This dose-lowering capability may become important in the management of adverse events by allowing patients to maintain a TKI which at higher concentrations would otherwise cause conditions indicated in the black box warning label.

Size, Potency, Delivery, and Conjugation

The remainder of this future directions section surrounds the pharmaceutical engineering to enhance the stability and delivery of the CC^{mut3} to the target cell. Here we will focus on moving away from gene-based therapy and toward a more readily-available and approachable delivery method.

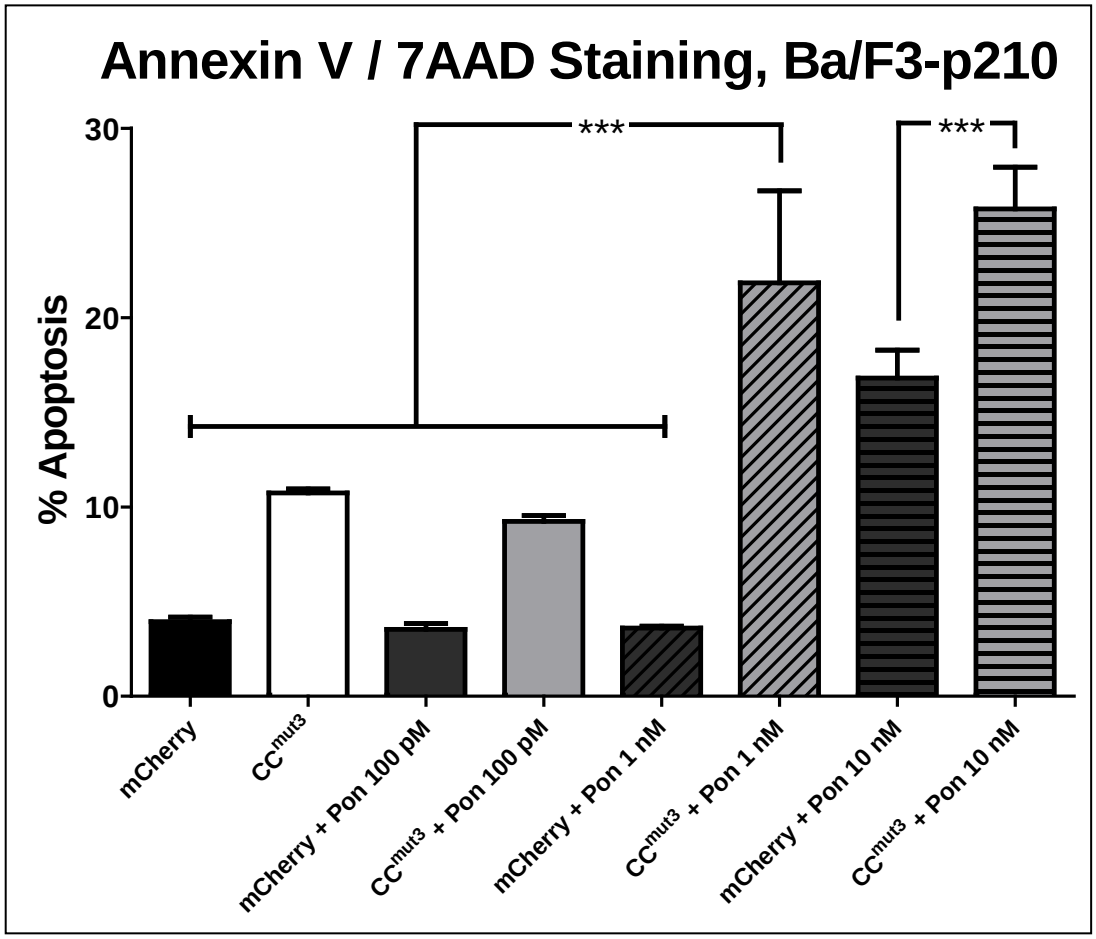

Figure 5.2 CC^{mut3} enables dose-lowering of ponatinib while maintaining apoptotic-induction potency. mCherry control or CC^{mut3} were transfected into Ba/F3 p210 cells, and then treated with increasing doses of ponatinib as indicated. Apoptosis was measured by flow cytometry after 72 h of exposure to both treatments.

Peptide Size: The Elephant in the Room

The full CC domain is comprised of two α-helices represented by 72 amino acids (AA), which we have continued to use for the CC^{mut2} and CC^{mut3}. This 72-AA mutated CC domain can efficiently disrupt dimerization of BCR-ABL1 homodimers to force a proapoptotic and antioncogenic phenotype (8, 9). The previous work on this domain suggested a truncation that has similar activity as our $CC^{mut2/3}$ (15). However, we observed a discrepancy between our results and those previously reported regarding the ability to inhibit BCR-ABL1-driven transformation from a T315I mutant (14). It is unclear at this point whether there is added stability of the α1-helix or if it is the rational manipulation of the α2-helix that confers this activity. Geoff Miller is currently working on truncation mutants to resolve these questions. It should be noted that the potential for instability likely increases as the size of the protein is shortened. During the early experiments for Chapter 4, an isolated 72-AA peptide delivered using lentivirus failed to potently express CC^{mut3}. It is unclear if RNA degradation occurred, or protein instability or increased degradation was the cause of this event (Figure 5.3). This problem was solved by fusing CC^{mut3} to EGFP for subsequent experiments. However, there are methods to improve the stability of small peptides which will be discussed in the following section.

Hydrocarbon Staples: Good for the CC^{mut3}-Inhibitory Environment

The CC^{mut3} is an ideal candidate for stapled-peptide technology. Peptide stapling can occur for many different motifs, but is uniquely suited for an α-helix (50). Peptide stapling provides stabilization for the desired motif through a hydrocarbon-conjugated

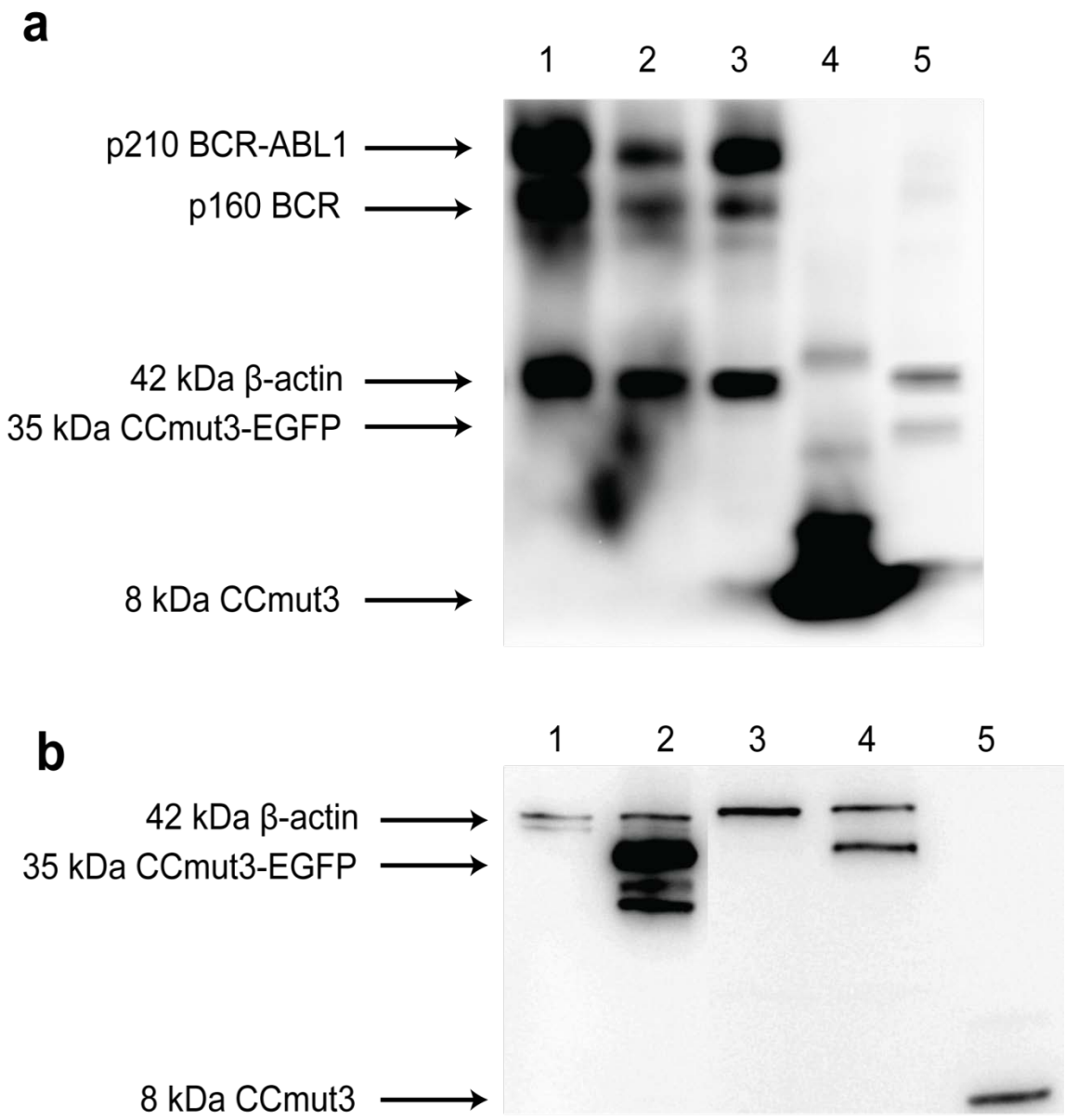

Figure 5.3 Stability of CC^{mut3} domain vs. CC^{mut3}-EGFP fusion in viral producing and K562 cells. (a) Virally delivered empty vector (1), CC (2), or CC^{mut3} (3) in K562 cells is not expressed at the corresponding size as should be detected by the BCR-N20 antibody, BCR and P210 BCR-ABL are evident in lanes 1-3. A positive control, purified CC^{mut3} peptide collected from bacteria cells is in lane 4. Lane 5 shows K562 cells transfected with the CC^{mut3}-EGFP fusion plasmid pEGFP-CC^{mut3} also show expected bands. (b) 293-FT virus-producing cells transfected with lentiviral plasmids expressing empty vector (1) or EGFP-CC^{mut3} (2). Virus harvested from cells run in lanes 1 and 2 was induced into K562 cells shown in lanes 3 and 4, with empty and EGFP-CC^{mut3} fusion, respectively. Lane 5 depicts the same 8 kDa purified CC^{mut3} as seen in Figure 5.3a, lane 5.

linker attached to the peptide backbone Figure 5.4 (51). Currently, Aileron Therapeutics holds the technology patent for peptide stapling, but novel peptides requiring stapling can be out-licensed. The advantage in using a stapled peptide goes beyond specificity to include limiting degradation, and increasing cell solubility (52, 53). Geoff Miller has identified some putative residues which may be good candidates for staple conjugation. These residues are limited to the "back" of the α-helix, and would not occupy the same residues where designed mutations exist (e.g., G29, Q33, E36) (Figure 5.5). Because this technique requires more synthetic chemistry, it would be ideal to synthesize the CC^{mut3} for this project. Overall yield and purity for synthetically produced proteins could be increased if the therapeutic α-helix were smaller, relating back to the previous series of proposed experiments regarding truncation.

Penetrating the Target

Other work in the Lim laboratory by Ben Bruno centers on incorporating a leukemia-specific cell-penetrating peptide (LS-CPP) into the CC^{mut3}. Work by Nishimura et al., described the specificity of a LS-CPP-containing the amino acid sequence CAYRLLR, which consists of a lymph node homing motif (CAY) and a cell-penetrating sequence (RLLR) (54). So far the LS-CPP-CC^{mut3} expression and purification from *E. coli* bacteria has been optimized, and we are beginning to test the uptake and dose-response of the LS-CPP-CC^{mut3} in apoptosis-inducing experiments.

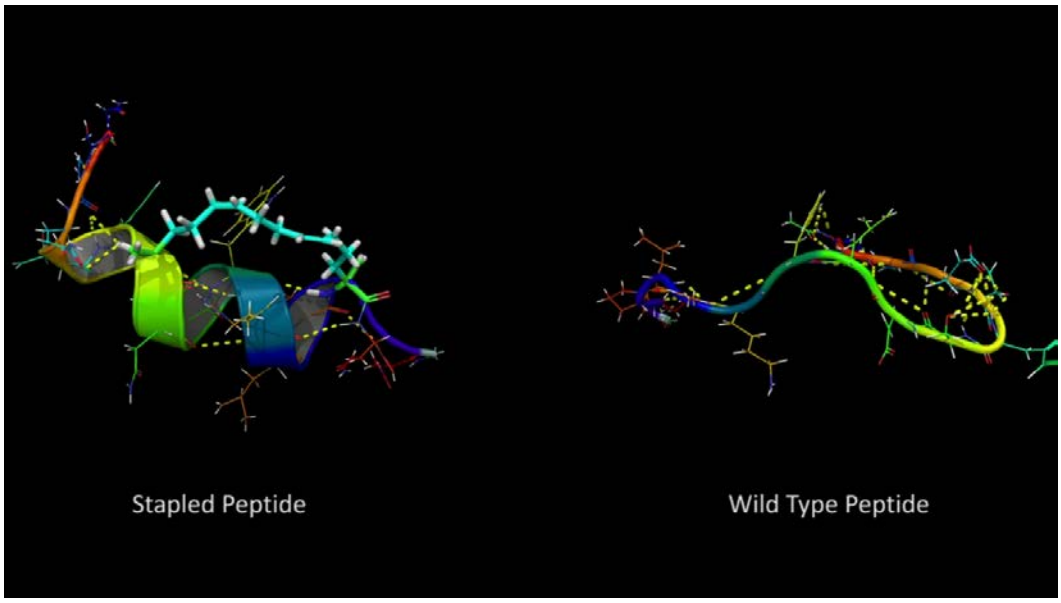

Figure 5.4 Differences in stability of stapled vs. nonstapled peptides. Here, a synthetic stapled peptide which mimics part of the p53-binding domain is compared to an identical nonstapled, wild-type peptide. This was a snapshot from a replica exchange molecular dynamics simulation using Schrodinger Suite 2009 and Pymol.

This frame from the YouTube video located at the following link was obtained with permission from the author, Noeris Salam.
http://www.youtube.com/watch?v=WScPbvUwDno

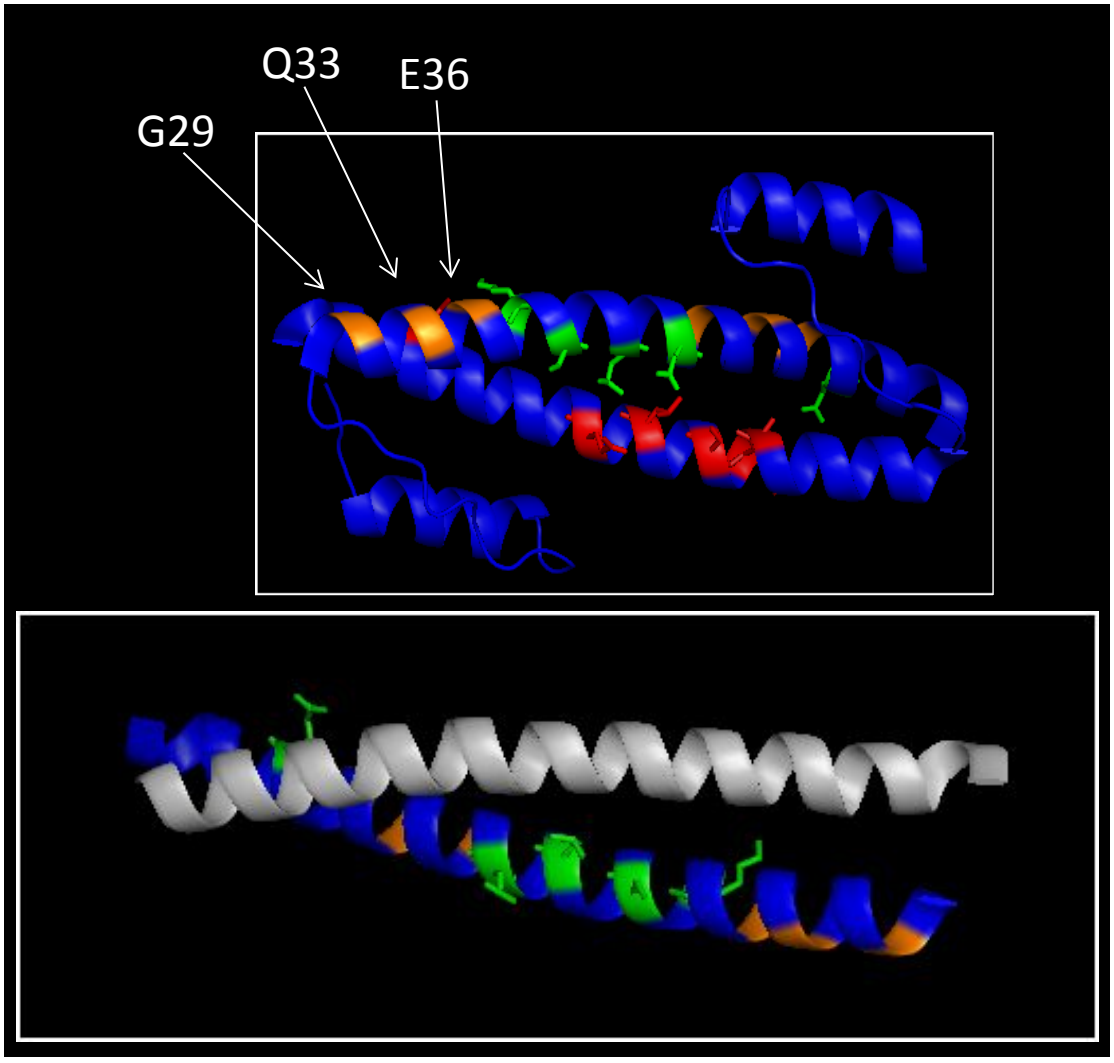

Figure 5.5 CC^{mut3} stapling locations. (Top) A ribbon diagram representing the entire CC, α-1 and α-2 helixes, is illustrated. The CC^{mut3} (with highlighted green side chains) is interacting with wild-type CC (red side chains). Orange highlighted amino acids represent potential peptide hydrocarbon stapling locations. (Bottom) The above diagram is turned on its side, with the α-1 helix removed for clarity. The wild-type CC is now in gray, while the CC^{mut3} is identical to that shown in the top panel.

Dimerization Motifs in Oncogenic Fusion Proteins

The BCR-ABL1 fusion protein and resulting therapeutic development of TKIs for disease management are a prototype for rational drug design. As we continue to show efficacy of the CC^{mut3} in this disease, we must also consider other disease-causing translocations which incorporate dimerization domains. A tangible example is non-BCR-ABL1 (Ph-like) ALL, which in some cases harbors a BCR-JAK2 protein fusion product responsible for enhanced kinase activity. This aberrant kinase activity cannot be ameliorated with either a JAK2 inhibitor (XL019) or dasatinib (55). When considering non-BCR fusions, these new dimerization domains may also be susceptible to rational design of a dimerization inhibitor as we did with the CC^{mut3}. While not all fusion products exhibit kinase activity, DNA interactions can equally drive oncogenesis after fusion by initiating transcription following dimerization (56). Examples of other dimerization domain-containing oncogenic fusion proteins include: the TFG-ALK (CC domain – kinase) fusion in lymphoma and non-small cell lung carcinoma (57); MLL-ENL (AT-hook domain – trans-activator helix) fusion in ALL (58); and EWS-ATF1 (EAD domain – leucine-zipper) in solid tumors and melanomas (59). Disruption of kinase activity, or DNA transcription could be specifically inhibited using dimerization disruption (similar to CC^{mut3}) rather than a kinase inhibitor, or by the myriad of anti-neoplastics which disrupt DNA transcription.

Summary

There is significant progress being made in the study of the CC^{mut3} as a cancer therapeutic with utility in Ph^{+} leukemias. If any or all of the above proposed

improvements to CC^{mut3} delivery prove to be successful, there is ultimately no reason not to combine them into a single therapeutic agent with the highest possible potency and specificity for BCR-ABL1 dimer inhibition. As work continues on this project, we hope to one day see this agent available clinically. To this end we have filed several invention disclosure forms with the University of Utah Technology Commercialization Office (TCO); however, at this time no patents have been filed by the University on our behalf. As stepwise improvements are made, we will continue to file for protection of intellectual property and strive to translate this from the bench to the bedside.

References

1. Nowell PC. The minute chromosome (phl) in chronic granulocytic leukemia. *Blut.* 1962;8(65-6.

2. Nowell PC, Jensen J, and Gardner F. Two complex translocations in chronic granulocytic leukemia involving chromosomes 22, 9, and a third chromosome. *Humangenetik.* 1975;30(1):13-21.

3. Kurzrock R, Shtalrid M, Talpaz M, Kloetzer WS, and Gutterman JU. Expression of c-abl in philadelphia-positive acute myelogenous leukemia. *Blood.* 1987;70(5):1584-8.

4. Kurzrock R, Shtalrid M, Romero P, Kloetzer WS, Talpas M, Trujillo JM, Blick M, Beran M, and Gutterman JU. A novel c-abl protein product in philadelphia-positive acute lymphoblastic leukaemia. *Nature.* 1987;325(6105):631-5.

5. Walker LC, Ganesan TS, Dhut S, Gibbons B, Lister TA, Rothbard J, and Young BD. Novel chimaeric protein expressed in philadelphia positive acute lymphoblastic leukaemia. *Nature.* 1987;329(6142):851-3.

6. Kurzrock R, Kantarjian HM, Druker BJ, and Talpaz M. Philadelphia chromosome-positive leukemias: From basic mechanisms to molecular therapeutics. *Ann Intern Med.* 2003;138(10):819-30.

7. Beissert T, Puccetti E, Bianchini A, Guller S, Boehrer S, Hoelzer D, Ottmann OG, Nervi C, and Ruthardt M. Targeting of the n-terminal coiled coil oligomerization interface of bcr interferes with the transformation potential of bcr-abl and increases sensitivity to sti571. *Blood.* 2003;102(8):2985-93.

8. Dixon AS, Pendley SS, Bruno BJ, Woessner DW, Shimpi AA, Cheatham TE, 3rd, and Lim CS. Disruption of bcr-abl coiled-coil oligomerization by design. *J Biol Chem.* 2011.

9. Dixon AS, Miller GD, Bruno BJ, Constance JE, Woessner DW, Fidler TP, Robertson JC, Cheatham TE, 3rd, and Lim CS. Improved coiled-coil design enhances interaction with bcr-abl and induces apoptosis. *Mol Pharm.* 2012;9(1):187-95.

10. Constance JE, Woessner DW, Matissek KJ, Mossalam M, and Lim CS. Enhanced and selective killing of chronic myelogenous leukemia cells with an engineered bcr-abl binding protein and imatinib. *Mol Pharm.* 2012;9(11):3318-29.

11. Health DPLNIo. Study safety and preliminary efficacy of dcc-2036 in patients with leukemias (ph+ cml with t315i mutation). *ClinicalTrials.gov [Internet].* 2013.

12. Cortes J, Talpaz M, Bixby D, Deininger M, Shah N, Flinn IW, Mauro M, O'Hare T, Hu S, Kan R, et al. A phase 1 trial of oral ponatinib (ap24534) in patients with refractory chronic myelogenous leukemia (cml) and other hematologic malignancies: Emerging safety and clinical response findings. *ASH Annual Meeting Abstracts.* 2010;116(21):210.

13. Khorashad JS, Kelley TW, Szankasi P, Mason CC, Soverini S, Adrian LT, Eide CA, Zabriskie MS, Lange T, Estrada JC, et al. Bcr-abl1 compound mutations in tyrosine kinase inhibitor-resistant cml: Frequency and clonal relationships. *Blood.* 2013;121(3):489-98.

14. Mian AA, Schull M, Zhao Z, Oancea C, Hundertmark A, Beissert T, Ottmann OG, and Ruthardt M. The gatekeeper mutation t315i confers resistance against small molecules by increasing or restoring the abl-kinase activity accompanied by aberrant transphosphorylation of endogenous bcr, even in loss-of-function mutants of bcr/abl. *Leukemia.* 2009;23(9):1614-21.

15. Beissert T, Hundertmark A, Kaburova V, Travaglini L, Mian AA, Nervi C, and Ruthardt M. Targeting of the n-terminal coiled coil oligomerization interface by a helix-2 peptide inhibits unmutated and imatinib-resistant bcr/abl. *Int J Cancer.* 2008;122(12):2744-52.

16. Corbin AS, Agarwal A, Loriaux M, Cortes J, Deininger MW, and Druker BJ. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of bcr-abl activity. *J Clin Invest.* 2011;121(1):396-409.

17. Nicholson E, and Holyoake T. The chronic myeloid leukemia stem cell. *Clin Lymphoma Myeloma.* 2009;9(S4):376-81.

18. O'Hare T, Eide CA, and Deininger MW. Bcr-abl kinase domain mutations, drug resistance, and the road to a cure for chronic myeloid leukemia. *Blood.* 2007;110(7):2242-9.

19. Mahon FX, Rea D, Guilhot J, Guilhot F, Huguet F, Nicolini F, Legros L, Charbonnier A, Guerci A, Varet B, et al. Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: The prospective, multicentre stop imatinib (stim) trial. *Lancet Oncol.* 2010;11(11):1029-35.

20. Yin L, Ahmad R, Kosugi M, Kawano T, Avigan D, Stone R, Kharbanda S, and Kufe D. Terminal differentiation of chronic myelogenous leukemia cells is induced by targeting of the muc1-c oncoprotein. *Cancer Biol Ther.* 2010;10(5):483-91.

21. Kawano T, Ito M, Raina D, Wu Z, Rosenblatt J, Avigan D, Stone R, and Kufe D. Muc1 oncoprotein regulates bcr-abl stability and pathogenesis in chronic myelogenous leukemia cells. *Cancer Res.* 2007;67(24):11576-84.

22. Trehin R, and Merkle HP. Chances and pitfalls of cell penetrating peptides for cellular drug delivery. *Eur J Pharm Biopharm.* 2004;58(2):209-23.

23. Suzuki T, Futaki S, Niwa M, Tanaka S, Ueda K, and Sugiura Y. Possible existence of common internalization mechanisms among arginine-rich peptides. *J Biol Chem.* 2002;277(4):2437-43.

24. Peng C, Chen Y, Shan Y, Zhang H, Guo Z, Li D, and Li S. Lsk derived lsk(-) cells have a high apoptotic rate related to survival regulation of hematopoietic and leukemic stem cells. *PLoS One.* 2012;7(6):e38614.

25. Al Baghdadi T, Abonour R, and Boswell HS. Novel combination treatments targeting chronic myeloid leukemia stem cells. *Clin Lymphoma Myeloma Leuk.* 2012;12(2):94-105.

26. Karandikar NJ, Aquino DB, McKenna RW, and Kroft SH. Transient myeloproliferative disorder and acute myeloid leukemia in down syndrome. An immunophenotypic analysis. *Am J Clin Pathol.* 2001;116(2):204-10.

27. Konopleva MY, and Jordan CT. Leukemia stem cells and microenvironment: Biology and therapeutic targeting. *J Clin Oncol.* 2011;29(5):591-9.

28. Jin L, Tabe Y, Konoplev S, Xu Y, Leysath CE, Lu H, Kimura S, Ohsaka A, Rios MB, Calvert L, et al. Cxcr4 up-regulation by imatinib induces chronic myelogenous leukemia (cml) cell migration to bone marrow stroma and promotes survival of quiescent cml cells. *Mol Cancer Ther.* 2008;7(1):48-58.

29. Woessner DW, Lim CS, and Deininger MW. Development of an effective therapy for chronic myelogenous leukemia. *Cancer J.* 2011;17(6):477-86.

30. Ponatinib: U.S. Food and Drug Administration. http://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm332368.htm. Accessed June 24, 2013.

31. Shah RR, Morganroth J, and Shah DR. Hepatotoxicity of tyrosine kinase inhibitors: Clinical and regulatory perspectives. *Drug Saf.* 2013;36(7):491-503.

32. Kantarjian HM, O'Brien S, and Cortes J. Homoharringtonine/omacetaxine mepesuccinate: The long and winding road to food and drug administration approval. *Clin Lymphoma Myeloma Leuk.* 2013;13(3):118-23.

33. Wetzler M, and Segal D. Omacetaxine as an anticancer therapeutic: What is old is new again. *Curr Pharm Des.* 2011;17(1):59-64.

34. El-Metnawy WH, Mattar MM, El-Nahass YH, Samra MA, Abdelhamid HM, Abdlfattah RM, and Hamed AR. Predictive value of pretreatment bcr-abl(is) transcript level on response to imatinib therapy in egyptian patients with chronic phase chronic myeloid leukemia (cpcml). *Int J Biomed Sci.* 2013;9(1):48-53.

35. Klamova H, Polakova KM, Muzik J, Racil Z, Zackova D, Steinerova K, Karas M, Faber E, Demeckova E, Michalovicova-Sninska Z, et al. Evaluation of 5-year imatinib treatment of 458 patients with cp-cml in routine clinical practice and prognostic impact of different bcr-abl cutoff levels. *Cancer Med.* 2013;2(2):216-25.

36. Akard LP, and Wang YL. Translating trial-based molecular monitoring into clinical practice: Importance of international standards and practical considerations for community practitioners. *Clin Lymphoma Myeloma Leuk.* 2011;11(5):385-95.

37. Ross DM, Branford S, Seymour JF, Schwarer AP, Arthur C, Yeung DT, Dang P, Goyne JM, Slader C, Filshie RJ, et al. Safety and efficacy of imatinib cessation for cml patients with stable undetectable minimal residual disease: Results from the twister study. *Blood.* 2013.

38. Russo D, Martinelli G, Malagola M, Skert C, Soverini S, Iacobucci I, De Vivo A, Testoni N, Castagnetti F, Gugliotta G, et al. Effects and outcome of a policy of intermittent imatinib treatment in elderly patients with chronic myeloid leukemia. *Blood.* 2013.

39. Kakar M, Davis JR, Kern SE, and Lim CS. Optimizing the protein switch: Altering nuclear import and export signals, and ligand binding domain. *J Control Release.* 2007;120(3):220-32.

40. Dixon AS, Constance JE, Tanaka T, Rabbitts TH, and Lim CS. Changing the subcellular location of the oncoprotein bcr-abl using rationally designed capture motifs. *Pharm Res.* 2012;29(4):1098-109.

41. Dixon AS, and Lim CS. The nuclear translocation assay for intracellular protein-protein interactions and its application to the bcr coiled-coil domain. *Biotechniques.* 2010;49(1):519-24.

42. Dixon AS, Kakar M, Schneider KM, Constance JE, Paullin BC, and Lim CS. Controlling subcellular localization to alter function: Sending oncogenic bcr-abl to the nucleus causes apoptosis. *J Control Release.* 2009;140(3):245-9.

43. Vigneri P, and Wang JY. Induction of apoptosis in chronic myelogenous leukemia cells through nuclear entrapment of bcr-abl tyrosine kinase. *Nat Med.* 2001;7(2):228-34.

44. Constance JE, Despres SD, Nishida A, and Lim CS. Selective targeting of c-abl via a cryptic mitochondrial targeting signal activated by cellular redox status in leukemic and breast cancer cells. *Pharm Res.* 2012;29(8):2317-28.

45. Al-Achkar W, Aljapawe A, Almedani S, Liehr T, and Wafa A. A novel cytogenetic abnormality t(7;8)(p11.2:Q11.2) and a four-way philadelphia

translocation in an imatinib mesylate-resistant chronic myeloid leukemia patient. *Oncol Lett.* 2013;5(2):617-20.

46. Oh D, Han S, Seo J, Lee JR, Choi J, Groffen J, Kim K, Cho YS, Choi HS, Shin H, et al. Regulation of synaptic rac1 activity, long-term potentiation maintenance, and learning and memory by bcr and abr rac gtpase-activating proteins. *J Neurosci.* 2010;30(42):14134-44.

47. Zhang X, Subrahmanyam R, Wong R, Gross AW, and Ren R. The nh(2)-terminal coiled-coil domain and tyrosine 177 play important roles in induction of a myeloproliferative disease in mice by bcr-abl. *Mol Cell Biol.* 2001;21(3):840-53.

48. Bradeen HA, Eide CA, O'Hare T, Johnson KJ, Willis SG, Lee FY, Druker BJ, and Deininger MW. Comparison of imatinib mesylate, dasatinib (bms-354825), and nilotinib (amn107) in an n-ethyl-n-nitrosourea (enu)-based mutagenesis screen: High efficacy of drug combinations. *Blood.* 2006;108(7):2332-8.

49. Sullivan C, Peng C, Chen Y, Li D, and Li S. Targeted therapy of chronic myeloid leukemia. *Biochem Pharmacol.* 2010;80(5):584-91.

50. Kutchukian PS, Yang JS, Verdine GL, and Shakhnovich EI. All-atom model for stabilization of alpha-helical structure in peptides by hydrocarbon staples. *J Am Chem Soc.* 2009;131(13):4622-7.

51. Walensky LD, Kung AL, Escher I, Malia TJ, Barbuto S, Wright RD, Wagner G, Verdine GL, and Korsmeyer SJ. Activation of apoptosis in vivo by a hydrocarbon-stapled bh3 helix. *Science.* 2004;305(5689):1466-70.

52. Bird GH, Madani N, Perry AF, Princiotto AM, Supko JG, He X, Gavathiotis E, Sodroski JG, and Walensky LD. Hydrocarbon double-stapling remedies the proteolytic instability of a lengthy peptide therapeutic. *Proc Natl Acad Sci USA.* 2010;107(32):14093-8.

53. Verdine GL, and Hilinski GJ. Stapled peptides for intracellular drug targets. *Methods Enzymol.* 2012;503(3-33.

54. Nishimura S, Takahashi S, Kamikatahira H, Kuroki Y, Jaalouk DE, O'Brien S, Koivunen E, Arap W, Pasqualini R, Nakayama H, et al. Combinatorial targeting of the macropinocytotic pathway in leukemia and lymphoma cells. *J Biol Chem.* 2008;283(17):11752-62.

55. Roberts KG, Morin RD, Zhang J, Hirst M, Zhao Y, Su X, Chen SC, Payne-Turner D, Churchman ML, Harvey RC, et al. Genetic alterations activating kinase and cytokine receptor signaling in high-risk acute lymphoblastic leukemia. *Cancer Cell.* 2012;22(2):153-66.

56. Hegyi H, Buday L, and Tompa P. Intrinsic structural disorder confers cellular viability on oncogenic fusion proteins. *PLoS Comput Biol.* 2009;5(10):e1000552.

57. Roccato E, Pagliardini S, Cleris L, Canevari S, Formelli F, Pierotti MA, and Greco A. Role of tfg sequences outside the coiled-coil domain in trk-t3 oncogenic activation. *Oncogene.* 2003;22(6):807-18.

58. Slany RK, Lavau C, and Cleary ML. The oncogenic capacity of hrx-enl requires the transcriptional transactivation activity of enl and the DNA binding motifs of hrx. *Mol Cell Biol.* 1998;18(1):122-9.

59. Pan S, Ming KY, Dunn TA, Li KK, and Lee KA. The ews/atf1 fusion protein contains a dispersed activation domain that functions directly. *Oncogene.* 1998;16(12):1625-31.